Tonsillar carcinomas in Norway, changes in etiology and prognosis

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LIST OF ORIGINAL PAPERS

1. Hannisdal K, Boysen M, Evensen JF.
Different prognostic indices in 310 patients with tonsillar carcinomas.
Head Neck 2003;25: 123-131

2. Hannisdal K, Schjolberg A, de Angelis PM, Boysen M, Clausen OP.
Human papillomavirus (HPV)-positive tonsillar carcinomas are frequent and have a favourable prognosis in males in Norway.
Acta Otolaryngol 2010;130: 293-299

3. Hannisdal K, Burum-Auensen E, Schjolberg A, de Angelis PM, Clausen OP.
Correlation between reduced expression of the spindle checkpoint protein BubR1 and bad prognosis in tonsillar carcinomas.
Head Neck 2010;32: 1354-1362

ABBREVATIONS

BLAST	Basic local alignment search tool
BubR1	Bubbing uninhibited by benzimidazole
CI	Confidence intervals
CIN	Chromosomal instability
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus
EGFR	Epidermal growth factor receptor
HNC	Head and neck cancer
HPV	Human papilloma virus
HR	Hazard ratio
ICD	International Classification of Disease
IHC	Immunohistochemistry
ISH	In situ hybridization
Mad2	Mitotic arrest deficient 2
OR	Odds ratio
PCR	Polymerase chain reaction
PCS	Premature chromatide separation syndrome
SAC	Spindle assembly checkpoint
SCC	Squamous cell carcinomas
SCCHN	Squamous cell carcinomas of head and neck
TC	Tonsillar carcinomas
TMA	Tissue microarrays
TNM	Tumor Node Metastasis staging system
UNPC	Undifferentiated nasopharyngeal carcinoma
UICC	Union Internationale Contre le Cancer
VEGF	Vascular endothelial growth factor
WHO	World Health Organization

INTRODUCTION

Overview of cancer in the head and neck region

Head and neck cancer (HNC) is the sixth most common cancer with about 650.000 new cases and estimated 350.000 cancer deaths worldwide every year.[8;43] Squamous cell carcinomas (SCC) are found in 90% of all HNC. HNC is a heterogeneous type of cancer and shows great variations among gender, age and ethnic groups. The international classification of diseases (ICD) promotes international comparability in collection, classification and presentation of incidence and mortality statistics. In a global perspective the incidence of HNC in Norway is low and constituted 690 of 25.577 (2.7%) of all new cancers in 2006. The distribution of locations of new cases in Norway in 2006 is shown in Table 1 (data from The Norwegian Cancer Registry) according to the ICD-O.

Table 1. Cancers in Norway in 2006

Locations	**New cases**			**Incidence rates per 100.000 (age adjusted)**		
ICD-O codes	***Males***	***Females***	***Total***	***Males***	***Females***	***Total***
C00-C96 All cancers	13410	12167	25577	343,5	288,9	310,9
C00 Lip	68	46	114	1,6	1,0	1,2
C01-C02 Tongue	47	39	86	1,2	0,9	1,1
C03-C06 Oral cavity	63	61	124	1,7	1,4	1,6
C07-C08 Salivary glands	17	32	49	0,4	0,8	0,6
C09 Tonsils	47	18	65	1,4	0,5	1,0
C10-C14 Naso,- oro-, hypopharynx	54	23	77	1,6	0,7	1,1
C30-31 Nasal cavity, sinus	24	23	47	0,6	0,6	0,6
C32 Larynx	116	12	128	3,2	0,3	1,7

Oropharynx and the tonsillar region

The oropharynx includes 4 areas:

- The base of the tongue
- The tonsillar area (tonsillar fossa and tonsillar pillars)
- The soft palate
- The portion of the pharyngeal wall between the pharyngoepiglottic fold and the nasopharynx.

Figure 1 and 2. Basic anatomical structures in the oropharynx and the tonsillar region.[98]

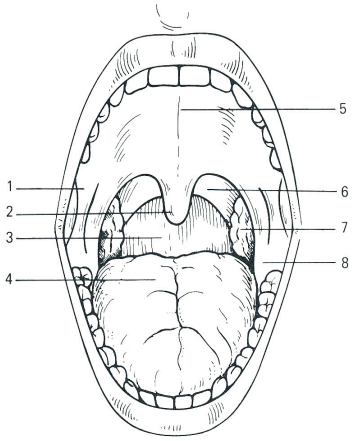


Figure 10.12 The mouth and oropharynx seen from in front

1. Pterygomandibular raphe
2. Uvula
3. Posterior pharyngeal wall
4. Tongue
5. Soft palate
6. Palatopharyngeal fold
7. Palatine tonsil
8. Palatoglossal fold

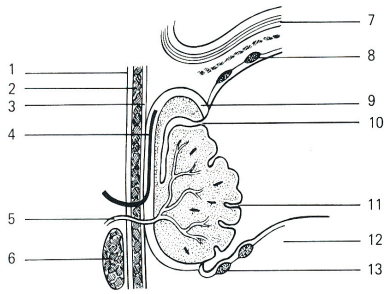


Figure 10.23 A diagram of a coronal section through the palatine tonsil to show local relationships

1. Buccopharyngeal fascia
2. Middle constrictor
3. Pharyngobasilar fascia
4. Paratonsillar vein
5. Tonsillar artery
6. Styloglossus
7. Soft palate
8. Lymphoid follicles
9. Tonsil capsule
10. Intratonsillar cleft
11. Tonsillar crypt
12. Base of tongue
13. Lingual tonsil

The palatine tonsil

The palatine tonsil is an almond-shaped mass of largely lymphoid tissue embedded in a fibrous capsule. It is situated in the triangular fossa between the diverging palatopharyngeal and palatoglossal folds (the tonsillar pillars). The medial portion of the tonsil is free and projects into the oropharynx. Laterally the floor of the tonsil is formed by the pharyngobasilar fascia deep to which, in the upper part

of the fossa are the superior constrictor muscles and below is the styloglossus muscle. The superior part of the tonsil is separated from the base of the uvula by a fold of mucous membrane from the palatopharyngeal arch. The inferior portion of the fossa is the glossopalatine sulcus.

Structure of the tonsil

The tonsil consists of a mass of lymphoid follicles in a connective tissue framework. The epithelial lining is a non-keratinizing stratified columnar epithelium. In the centre of each nodule, the germinal centre, the lymphocytes are less packed and here the multiplication of the lymphocytes takes place. The medial surface of the tonsil constitutes 15-20 openings irregularly spaced over the surface. These openings leading to deep, narrow, blinded recesses are termed the tonsillar crypts. The crypts distinguish it from other lymphoid organs because they may penetrate almost the whole thickness of the tonsil. The lateral part of the tonsil is not covered by a mucous membrane, but a fibrous capsule separating the tonsil from the wall of the oropharynx by loose areolar tissue. After tonsillectomy the whole tonsillar fossa is lined by stratified epithelium.

Nerve and blood supply

The tonsillar branch of the glossopharyngeal nerve is the main sensory nerve supply. The upper part of the tonsil is supplied by the lesser palatine nerves, branches of the maxillary division of the trigeminal nerve. Sympathetic fibres reach the tonsil on arteries supplying it and come from the superior cervical ganglion. The main artery is the tonsillar branch of the facial artery. Further arterial supply is from the lingual artery and the greater palatine vessels from the maxillary artery. The venous drainage is to the paratonsillar veins and vessels passing to the pharyngeal plexus or facial vein.

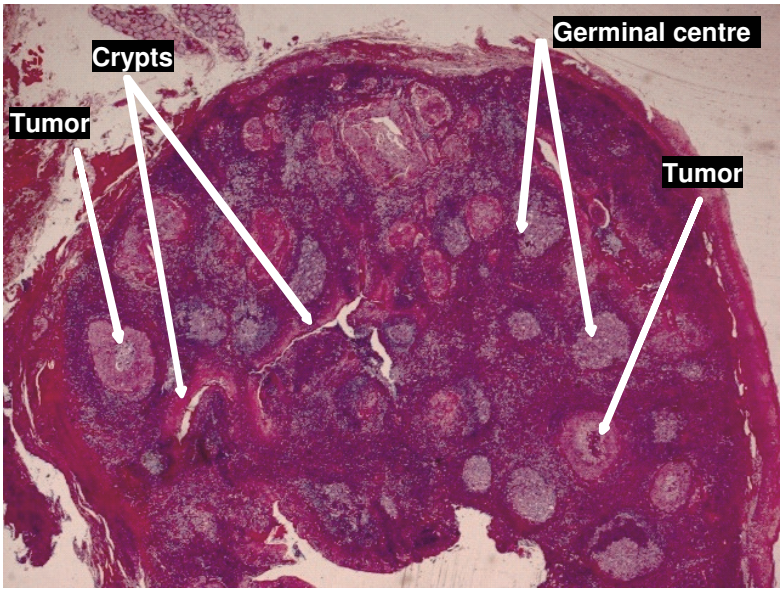
Lymphatic drainage

Lymphatic vessels pass to the upper deep cervical group of nodes, particularly the jugulodigastric group situated just below the posterior belly of the digastric muscle.

Histopathology and staging

SCC are the most common of tonsillar neoplasms (94%), while malignant lymphomas account for about 5% of tonsillar malignancies. Plasmacytomas, sarcomas and other rare tumors may occur in the tonsillar area. SCC may be classified by degree of differentiation into well-, moderately- and poorly-differentiated types.

Figure 3. Tonsil with SCC



In larger tumors (> T1) it may be difficult to discriminate between an origin in the tonsillar fossa versus an origin in the tonsillar pillars or the rest of the oropharynx. Therefore our series included all C09 locations (or the ICD-7 locations 145.0-145.9 as coded in the Norwegian Cancer Registry). Tumors of the tonsillar fossa are either exophytic or ulcerative and are generally present in a more advanced stage than are tumors of the tonsillar pillars. Lesions involving the anterior tonsillar pillar may appear as areas of dysplasia, inflammation or exophytic lesions and they often ulcerate.

Epidemiology and risk factors

The incidence of cancer at the base of the tongue and the tonsils has increased worldwide, especially in males and age less than 45 years.[38] The increase in incidence of tonsillar cancer over decades in Norway is shown in Table 2 (data from the Norwegian Cancer Registry) and males have the most marked increase in incidence.

Table 2. Incidence of tonsillar cancer in Norway in three different time periods (only palatine tonsil, ICD-O: C09.9)

	New cases			Incidence rates per 100.000 (age adjusted)		
	Males	***Females***	***Total***	***Males***	***Females***	***Total***
1960-1984	242	93	335	0.3	0.1	0.2
1985-1996	224	87	311	0.7	0.2	0.4
1997-2006	306	102	408	1.0	0.3	0.6

Social inequalities are related to the risk of HNC. Individuals with low education, low social class or low income have an odds ratio (OR) of 1.8-2.4 for developing HNC compared to others.[40] HNC has been strongly linked to chronic tobacco and alcohol abuse. Tobacco consumption as a risk factor for HNC was recently reported in a metaanalysis of 10 studies. For current smokers the OR was 7.[60] Involuntary smoking (passive smoking) is also associated with an increased risk for HNC, with an OR of 1.6 after exposure of more than 15 years at home.[60] Frequent alcohol consumption increases the risk of cancers of the oropharynx.[77] More than 350 g of alcohol per week with an OR of 2.6 and 11-20 cigarettes per day (OR 2.4) were dose-dependent risk factors. The results showed a tendency for women to have a greater risk (OR 1.8) than men at any given level of tobacco consumption.[151]

Quitting tobacco or alcohol use has been reported to reduce the HNC risk in several studies. In a large metaanalysis, persons who quit tobacco smoking for 1-4 years had a cancer risk reduction (OR 0.7) compared with current smokers. Smoking cessation for 20 years or more gave a further risk reduction reaching the level of never smokers (OR 0.2). For alcohol use a beneficial effect on the risk of HNC was only observed after >20 years of quitting (OR 0.6 compared with current drinking), reaching the level of never drinkers. These results support that cessation of tobacco smoking and

cessation of alcohol drinking protect against the development of HNC.[120] Tobacco chewing is associated with higher risk for HNC. Betel quid is carcinogenic for the oral cavity and hypopharynx.[43]

About 15-20% of HNC occurs in non-smokers and non-drinkers, suggesting the presence of other risk factors.[63] In Japan tooth loss as a result of poor oral hygiene, has been found to be associated with higher risk for HNC.[86] In a case control study of 132 patients with oral and oropharyngeal SCC in Sweden, oral hygiene, dental status, alcohol and tobacco use and human papilloma virus (HPV) infection were risk factors for developing cancer. Individuals who have a low body mass index also have an increased risk of HNC. This may be explained by increased effects of carcinogens in patients with low body mass index.[139] Diet has a role for developing HNC, and an inverse association exist between total fruit and vegetable intake and the risk of HNC.[43]

HPV is a member of the papilloma virus family capable of infecting humans and causing cancer.[182] HPV establishes productive infections only in the mucous membranes covered by squamous epithelium of the skin. A minority of the nearly 200 known types of HPV leads to cancers. HPV is thus subdivided into two main groups, the high risk and the low risk viruses, referring to their ability to cause cancer. In the high risk group HPV-16 is the most common type found in tonsillar carcinomas (TC).[170]

An association between HPV and oral carcinomas was first reported in 1983,[169] and in 1989 HPV-16 DNA was reported to be present in TC.[24] Gillison et al presented in 2000 a series of 253 squamous cell carcinomas of head and neck (SCCHN), where 25% were HPV-positive. The HPV-positivity at the three most frequent sites were oropharynx; 57%, oral cavity; 12% and hypopharynx 10%.[63] Mork et al published in 2001 an important case-control study (292 SCCHN compared to 1568 matched controls) from a joint Nordic cohort where it was shown that HPV exposure preceded development of clinical disease. HPV-16 seropositive patients had an increased risk of subsequent oropharyngeal cancer (OR 2.2). Tumor tissue analyses in 160 of these patients revealed that 50% of oropharyngeal- and 16% of oral SCC contained HPV-16.[129] In a Swedish study 3/320 healthy controls had HPV high risk DNA in mouthwash or tonsillar fossa.[74] Dahlstrøm et al found that “never smokers” and “never drinkers” with HNC most frequent were women, and more than half the “never smokers” and “never drinkers” patients with an oropharyngeal primary were serologically positive for HPV-16.[47] HPV-positive TC have been found significantly less often among tobacco smokers and/or tobacco chewers than in non-smokers and/or non-chewers.[84] In a case-control study oral HPV infection was

strongly associated with oropharyngeal cancer among subjects with or without the established risk factors of tobacco and alcohol use.[45]

In a review published in 2004 of 432 patients (27 patient series) with TC, 51% were HPV-positive, with HPV-16 being the most prevalent subtype (84%).[170] The worldwide geographical variations of HPV-positivity in TC are great. In Taiwan only 13% of TC cancer were found to be HPV-positive,[36] in Hong Kong 29% (1985-2004) and in Australia 46% (1990-2001).[113] During the period 1986-2007, 43% of Greek TC patients had HPV in their tumors.[150] In Middle Germany for the period 1996-2005 76% of the TC were HPV-positive.[65] The highest HPV prevalence in TC in the world is reported from Sweden. In the county of Stockholm, 93% of TC were HPV-positive in the period 2006-2007.[130]

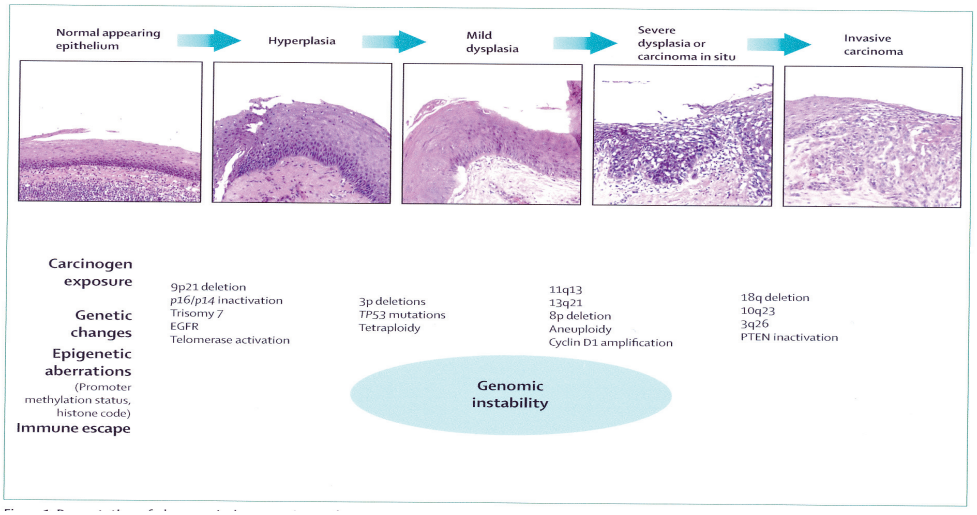
The estimates of HPV prevalence vary with the locations of tumors within the head and neck region, and tumors in the tonsillar region have a remarkably higher viral load compared to carcinomas at other sites.[35] Hobbs et al systematically reviewed studies that tested for HPV-16 exposure in anatomically defined sites in the head and neck and in a control group. The association between HPV-16 and cancer was strongest for tonsil (OR 15.1), intermediate for oropharynx (OR 4.3) and weakest for oral (OR 2.0) and larynx (OR 2.0). Less than 5% of laryngeal carcinomas are HPV-positive.[87] The causal role of HPV in sino-nasal SCC is also emerging.[171] In summary, HPV is a well established risk factor for cervical-, anogenital cancer and HNC. The relation between HPV and vaginal-, vulvar-, penile-, skin-, oesophageal-, stomach-, lung-, breast-, bladder- and prostate cancer is not yet completely resolved.[158;182]

The HNC type showing the most consistent worldwide association with Epstein-Barr virus (EBV) is the undifferentiated form of nasopharyngeal carcinoma (UNPC). UNPC is characterized by the presence of undifferentiated carcinoma cells together with a prominent lymphocytic infiltrate, the latter is believed to be important for the growth of the tumor cells. A link between EBV and UNPC was suggested as early as 1966 based on serological studies.[181] However, EBV does not seem to be an important risk factor in TC. In 46 TC patients with T2N2bM0, EBV was only found in one case.[37]

Pathogenesis, phenotypic and genotypic aberrations in HNC

SCCHN originate from keratinized epithelial cells of the mucous lining. In Figure 4 a progression model for SCCHN developed by several works is shown.[8;29;138] Differences between normal epithelium and malignant cells of SCCHN are results of specific alterations in genes controlling deoxyribonucleic acid (DNA) repair, proliferation, apoptosis, invasion and angiogenesis. These changes are results of oncogene activation or tumor suppressor gene inactivation resulting from interaction with known risk factors in SCCHN carcinogenesis.

Figure 4. A progression model for SCCHN carcinogenesis.[8]



One of the most common events is inactivation of the TP53 suppressor gene which is found in about 50% of SCCHN. The TP53 gene is localized to chromosome region 17p13.1, encoding for the p53 protein which is directly involved in the regulation of the cell cycle. Loss of function of TP53 is common in human cancers, and inactivation of the TP53 gene via mutations during tumorigenesis may result in inappropriate progression through the cell cycle after DNA damage, thus resulting in survival of cells that otherwise might have been destined to die. Accumulation of p53 which most often results from mutations is associated with metastatic disease or poor prognosis in several cancer types.

The p16 gene is a tumor suppressor gene involved in cell cycle control. Inactivation of p16 is an early detectable change in SCCHN and leads to cellular proliferation and development of cancer.[166] Furthermore, loss of chromosome region 9p21 is found in 70-80% of SCCHN. Telomerase assists in telomere maintenance and immortalisation, and is reactivated in 90% of SCCHN.[122] Prognosis for patients with telomerase-negative tumors is worse than for those with telomerase-positive tumors.[104] MicroRNA is a class of post-transcriptional regulators. They are nucleotide RNA sequences that bind to complementary sequences in target mRNAs, usually resulting in their silencing. The contribution of microRNAs to carcinogenesis in SCCHN seems clear, and a study of 169 SCCHN showed that alterations in microRNA expression were related to exposures such as causal alcohol consumption.[9] In 187 patients with SCCHN, amplification of the MYC gene, which is an oncogene, was associated with tumor progression.[18]

Epidermal growth factor receptor (EGFR) is central to SCCHN biology. EGFR can trigger pathways that regulate cell proliferation, apoptosis, metastatic potential and angiogenesis. Increased EGFR expression is seen in 90% of SCCHN.[8] The process of new blood vessel formation, angiogenesis, is fundamental for the growth of tumors and development of metastasis. Angiogenesis is regulated by many factors; the most important are vascular endothelial growth factor (VEGF) and its receptors. In a study of 85 TC a significant inverse relationship between EGFR and HPV status was found.[56] VEGF and EGFR were risk factors for local recurrence and disease-specific death in univariate analyses, but the associations weakened after adjustment for HPV. Among patients treated with radiotherapy, VEGF was associated with disease-specific death after adjusting for HPV and TNM stage. Patients with tumors positive for EGFR and high levels of VEGF expression had a worse prognosis compared to all other groups combined after adjusting for HPV and TNM stage.[56] In 82 patients with SCCHN, HPV status correlated inversely with EGFR expression. EGFR overexpression was a negative prognostic factor regardless of HPV status, and HPV status was a prognostic factor for progression and survival.[100] In 135 oral SCC patients EGFR was not correlated to prognosis, in contrast to p53 and p16.[157] In 56 patients with oral and oropharyngeal carcinomas, VEGF was found to be a prognostic factor and VEGF positivity was associated with poor relapse-free and overall survival.[164]

DNA aneuploidy means numerical or structural chromosomal abnormalities and nearly all solid tumors have aneuploid cell clones. During cellular division whole chromosomes or fractions of

chromosomes are lost, which may result in the formation of aneuploid cells. DNA aneuploidy is found in TC and is associated with worse prognosis.[124] Chromosomal instability (CIN) implies that cancer cells loose or gain chromosomes or chromosomal material during mitosis representing an increased rate of change in the chromosomal structure,[110] and is associated with aneuploidy. Some researchers claim that aneuploidy develops late in cancer and is not a cause of cancer, but in many studies CIN is regarded as one of the earliest steps in human carcinogenesis[112;161] and is also found in HNC.[174] When cells become aneuploid there is an uneven distribution of genomic DNA to daughter cells during mitosis. The mechanisms underlying chromosomal instability and the development of aneuploidy however are still unknown.[121]

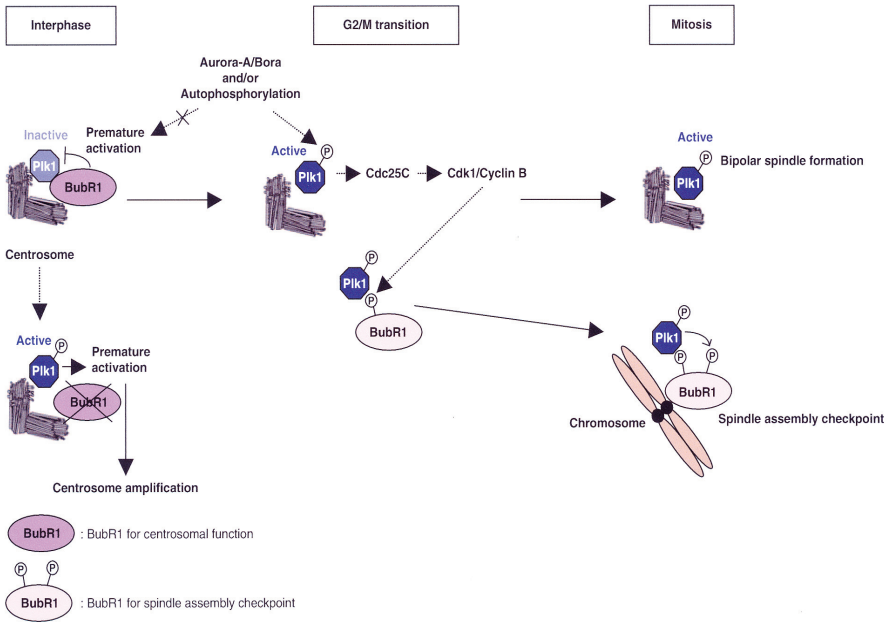
The spindle assembly checkpoint

The spindle assembly checkpoint (SAC) is a regulatory system that restrains progression through the metaphase-to-anaphase transition marked by sister chromatid separation. SAC delays anaphase until all sister chromatid pairs have become bipolarly attached. When the microtubulus attach to kinetochores (protein structures on chromosomes where the spindle fibres attach during division to pull the chromosomes apart), chromosomes are aligned on the metaphase plate and the SAC stopping mechanisms are removed. Thus the SAC can be seen as a house porter.

Our work has focused on two important proteins of the spindle checkpoint, Mad2 (Mitotic arrest deficient 2, Mad2L1 is the official gene name) and BubR1 (Bubbing uninhibited by benzimidazole, Bub1B is the official gene name),[92] which are activated by the lack of microtubule attachments to chromosomal kinetochores during metaphase.[102] The genetic regions coding for Mad2 and BubR1 are localized to the long arms of chromosome 4 (4q27) and chromosome 5 (5q14-21), respectively. BubR1 localizes to the kinetochores in early metaphase and prevents premature separation of sister chromatids. BubR1 also performs several roles during mitosis, mitotic timing and spindle function, but the interdependence of these functions is unclear.[146] Defective BubR1 plays a role in the regulation of apoptosis and chromosomal instability.[5;61]

Premature chromatide separation syndrome (PCS) is a rare autosomal recessive disorder characterized by premature separation of sister chromatids of all chromosomes.[90] Children with PCS show several abnormalities, including a high risk of malignancy, such as Wilms tumor and rhabdomyosarcoma. In PCS cells show severe chromosomal instability not only because of the mitotic checkpoint defect, but also because of the centrosome amplification. BubR1 deficiency causes centrosome amplification as well as SAC defects. This implies a novel role of BubR1 in preventing centrosome reduplication in interphase cells.[90] These findings are illustrated in Figure 5.

Figure 5. A model of some functions of BubR1.[90]

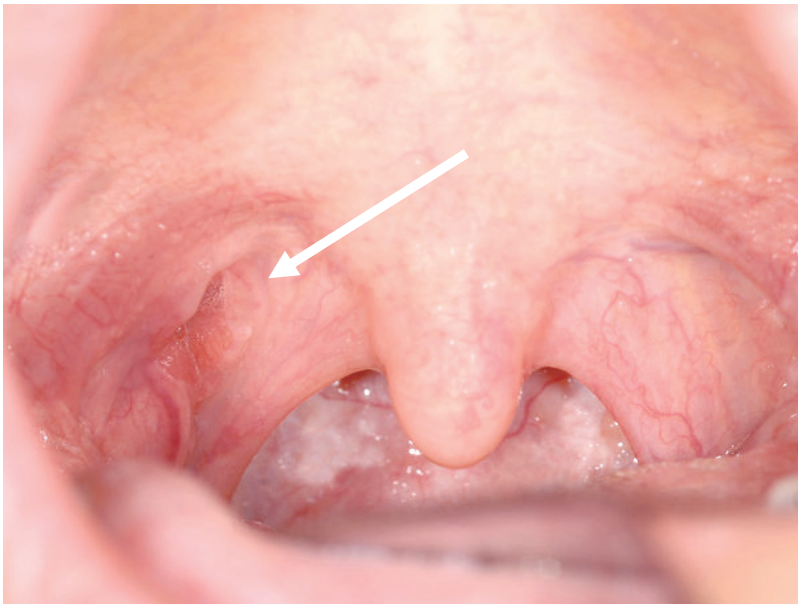


Several reports suggest that aberrant mitotic SAC proteins may be an important cofactor in the development of CIN and cancer development.[102] SAC defects and CIN were demonstrated in SCCHN in 2003,[128] and these cancers harbour defects in other genomic loci critical in tumor development and SAC control such as p53.[57] The role of BubR1 in HNC and its clinical significance is unclear. BubR1 protein is suggested to be one of the contributing factors involved in the pathogenesis of oral SCC, and is a possible marker for human oral squamous cell carcinogenesis.[89]

Clinical aspects

Patients with cancer in the oropharynx are often asymptomatic until their primary tumor reaches a significant size (T3, T4) or metastasizes to a lymph node in the neck (N+). The most common locations of cancer in the oropharynx are the tonsil and the anterior tonsillar pillar. Common presenting symptoms are ipsilateral referred otalgia, discomfort, dysphagia, sensation of a lump or foreign body in the throat, trismus, pain, tendency of aspiration or unpleasant odour. Biopsy of the primary tumor confirms the diagnosis. Suspected lymph nodes in the neck should be examined with cytological aspiration. In our patient series 65% had positive lymph nodes. The diagnosis of a positive lymph node was in the beginning of this study period based on clinical findings, since cytological aspiration was not an established method at that time. Distant metastases are seldom detected at diagnosis (2% in our population), but a complete staging process includes examination for metastatic disease.

Figure 6. An ulcerative T1 TC arising from the right tonsillar fossa



Treatment

Therapies available for the management of HNC include surgical resections, radiotherapy and chemotherapy. HNC has complex anatomic and physiologic relationships to the structures from which they arise. Multimodal management is required for advanced stage disease, while single modality treatment is usually sufficient for early lesions. Non-surgical therapies have been reevaluated in a metaanalysis of 6400 patients with oropharynx cancer (51 retrospective studies) who underwent (1) surgery with or without radiotherapy or (2) radiotherapy with or without neck dissection. The overall outcomes were the same, but the rate of severe complications was as high as 23% in the surgical subjects. The authors concluded that primary non-surgical treatment should be advocated.[137] Increased use of radiation, systemic/targeted therapies and function-preserving surgical approaches have allowed for organ preservation without compromising outcomes in properly selected patients.[107]

Radiotherapy

The treatment strategies for TC have changed over the years. From 1960-2000 radiotherapy was the mainstay of treatment. In radiotherapy the total dose and fractionation are most important. In the early megavoltage years, the 1950s and 1960s, the recommended dose for the tonsillar region was 60 Gy. Later the total dose raised to 65-70 Gy.[126;159] However, no clear time-dose relationship was apparent and research on dose-intensity emerged through the 1990s on the basis of the studies of Withers et al.[176] The dose-intensity can be altered through accelerated and/or hyperfractionated radiotherapy.[21] In accelerated regimens the total treatment time is reduced using 6 fractions per week. However, acute morbidity is significantly more intense with 6 than with 5 fractions per week.[135] This 6-fractions-weekly regimen has now become the standard treatment in HNC in Norway. In hyperfractionated radiotherapy two or three fractions are delivered each day (dose per fraction 1.1-1.5 Gy) to a higher total dose (76-81 Gy). In a metaanalysis of 15 trials with 5221 HNC patients, hyperfractionation had the greatest benefit with a 8% survival benefit at 5 years, while accelerated regimens without total dose reduction, had a 5-year survival benefit of 2% as compared to standard fractionation.[21] Mostly for practical reasons, hyperfractionation in HNC is not routine in Norway today.

Several studies have justified the role of radiotherapy as a primary treatment modality in early TC.[32] In addition, salvage surgery and neck dissections are important. The target for radiotherapy is the

primary tumor area and regional lymph nodes in the neck. The latter is included as a standard also when lymph node metastases are not diagnosed, due to the high risk of microscopic disease. For patients with involvement of lymph nodes in the neck, a neck dissection is usually performed 5-6 weeks after radiotherapy. Often these lymph nodes show no residual tumor after concomitant chemoradiotherapy.[118] The necessity of performing neck dissection after adequate radiotherapy has not been assessed in randomized clinical trials.

Chemoradiotherapy

The treatment paradigm for locally advanced HNC has evolved over the last decade as the role of chemotherapy has been substantiated by clinical trials. The superiority of cisplatin-based chemoradiotherapy in improving survival when compared with conventional radiotherapy alone in locally advanced SCC, has been documented in metaanalyses and clinical trials.[16;141;142] Concomitant cisplatin seems to give a 5-year survival benefit of 8%. In Norway, concomitant cisplatin-based chemoradiotherapy (one weekly dose of cisplatin up to 6 times) is now standard treatment for patients with unresectable disease (stage III and IV). The addition of a taxane in induction chemotherapy may improve efficacy over cisplatin and 5-FU,[178] but has not yet become a standard therapy.[142]

Elderly patients

The percentage of elderly people with HNC is rising due to increasing average lifespan. As with younger patients, elderly patients require a multidisciplinary approach in order to optimise treatment results. The biological- and not the chronological age should be defined individually based on co-morbidities and performance status. The different treatment modalities for HNC have been reported to be well tolerated by the elderly patients.[22] However, as discussed later, comorbidity and competing mortality are of increased importance in elderly patients.

New approaches in therapy

Novel therapeutic approaches such as immunotherapy are under clinical investigation. Emerging forms of HNC immunotherapy involve both the use of monoclonal antibodies that target growth factor receptors where immune activation appears to contribute to tumor cell lysis, as well as various forms of active vaccination strategies which activate and direct the patients cellular immunity against the tumor.[49]

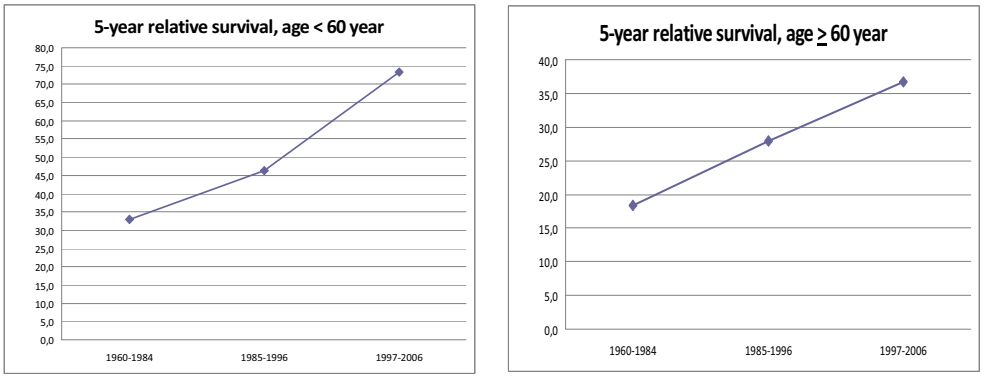
Anti-EGFR monoclonal antibodies, either as single agents or associated with chemotherapy, have been shown to be active and only slightly toxic.[8] Among them, cetuximab has proven to be the most promising. Cetuximab is an IgG1 monoclonal antibody against the ligand binding domain of EGFR. It enhances the cytotoxic effect of radiation in SCCHN. Bonner et al showed that the addition of cetuximab to high dose radiotherapy increased the control of locoregional disease and survival in 424 patients with stage III or stage IV SCC of the oropharynx, hypopharynx and larynx.[20] As compared to cisplatin-based chemotherapy plus fluorouracil alone in a randomized study of 442 patients, cetuximab plus cisplatin–fluorouracil chemotherapy improved overall survival when given as first-line treatment in patients with recurrent or metastatic SCCHN.[175] The question has been raised if cetuximab should replace cisplatin in HNC. However, a trial testing radiotherapy plus cetuximab versus radiotherapy plus cisplatin-based regimen has not been performed.[153]

For patients with HNC tumor hypoxia is a potent predictor of adverse outcomes.[133] Hypoxic cell radiosensitizer (nimorazole) given in association with conventional radiotherapy showed to be an important prognostic factor for locoregional control and survival in invasive carcinoma of the supraglottic larynx and pharynx.[134] HNC that is both hypoxic and highly angiogenic has a poor prognosis even after chemoradiotherapy, and patients with substantial overexpression of VEGF have a two-fold higher risk of dying. EGFR is abnormally activated in epithelial cancers and radiation increases the expression of EGFR. EGFR signalling also stimulates angiogenesis, but via mechanisms independent of hypoxia. Inhibition of one pathway (e.g. EGFR) probably upregulates signalling of alternative pathways, e.g.VEGF.[25] In a phase I/II study an EGFR inhibitor, erlotinib, was combined with an anti-VEGF antibody, bevacizumab, in patients with recurrent or metastatic SCCHN. Four of 48 patients had complete responses.[39]

Prognosis

The 5-year relative survival of patients with tonsillar cancer in Norway during three time periods is shown in Figure 7. Relative survival means survival rates that have been adjusted for other causes of death (competing mortality). As presented, the prognosis has improved significantly over the past decades in Norway. The largest difference is seen in patients with age < 60 (from 33% to 73%), while the 5-year relative survival for age ≥ 60 increased from 18% to 37%.

Figure 7. The 5-year relative survival (%) of patients with tonsillar cancer in Norway during three different time periods (ICD7: 145.0, ICD-O: C09.9)



Tumor-related prognostic factors

The most used staging system for TC is Tumor Node Metastasis staging system (TNM), coordinated by the International Union Against Cancer (UICC). The TNM system was introduced in 1943, but it was not until 1977 that HNC was included in this staging system. The staging system describes the anatomic extent (spread) of cancer before treatment. It was designed to aid in treatment planning and evaluation, to ease the communication between different treatment centres and to give some indication of prognosis.[83]

The TNM classification of 1978 for oropharyngeal cancer was:[75]

T0: No evidence of primary tumor

T1: Tumor 2 cm or less in greatest dimension

T2: Tumor more than 2 cm but no more than 4 cm in greatest dimension

T3: Tumor more than 4 cm in greatest dimension

T4: Tumor invades adjacent structures, e.g. through cortical bones, soft tissues of neck, deep muscles of tongue

N0: No evidence of regional lymph node involvement

N1: Evidence of involvement of movable homolateral regional lymph nodes

N2: Evidence of involvement of movable contralateral regional lymph nodes or bilateral lymph nodes

N3: Evidence of involvement of fixed regional lymph nodes

M0: No distant metastasis

M1: Distant metastasis present

In the revised TNM classification of 1989[168] the definitions of N categories for oropharyngeal cancer were modified as follows:

N0: No evidence of regional lymph node involvement

N1: Metastasis in a single ipsilateral lymph node, 3 cm or less in greatest dimension

N2a: Metastasis in a single ipsilateral lymph node, more than 3 cm but not more than 6 cm in greatest dimension

N2b: Metastasis in multiple ipsilateral lymph nodes, none more than 6 cm in greatest dimension

N2c: Metastasis in bilateral or contralateral lymph nodes, none more than 6 cm in greatest dimension

N3: Metastasis in lymph nodes, more than 6 cm in greatest dimension

In order to obtain a more balanced distribution of patients and thereby improve the prognostic information, several investigators have introduced new staging systems for HNC. These revised staging systems are all based on the established TNM system and do not deal with other variables. Hart and Berg regrouped TNM into 4 new stages generally relocating the patients toward more advanced stages.[15;76] Jones and co-workers introduced TANIS consisting of 7 new categories obtained by adding the integer values of T and N yielding a total T+N sum ranging from 1-7.[95] These categories were further regrouped into three stages. Later Snyderman regrouped TANIS into 4 stages.[167] Hall and co-workers proposed a system with 5 new prognostic levels where the N stage was modified combining N1 and N2a into N limited and N2b, N2c and N3 into N extended.[73]

Patient-related prognostic factors

The prognostic importance of the different patient variables has been explored in many patient series. A matched-pair analysis of race or ethnicity in outcomes of HNC patients receiving similar multidisciplinary care, showed no impact of race or ethnicity on survival.[33] Older HNC patients seem to have worse prognosis than younger patients,[94;106] but contrasting findings exist.[71] The impact of gender on prognosis in HNC is more unclear. Competing mortality in HNC is correlated to several patients factors and is higher in females.[123] In 226 and 1881 patients with SCC in the oropharynx, females have higher loco-regional control than males,[14;114] but gender was not a significant predictor of survival.[114] Hall et al did not find any prognostic importance of gender in 637 HNC patients,[73] and similar findings have been published for 81 TC patients[70] and 289 HNC patients.[93]

The performance status (Karnofsky, WHO, ECOG) allows patients to be classified according to their functional impairment. It can be used to compare effectiveness of different therapies and to assess the prognosis in individual patients.[41;72;93] Unfortunately, this variable often has many missing registrations in medical records. In 270 HNC patients a pretreatment hematologic profile was found to be a useful prognostic marker in patients with HNC. Both monocytosis, anemia and thrombocytosis had cumulative, negative effect on the prognosis.[34] A metaanalysis examined the evidence for an association between patient and/or provider-related diagnostic delay and late stage at diagnosis in 27 eligible studies. The relationship between diagnostic delay and stage at diagnosis varied in direction and magnitude, with no consistent positive association in any of the HNC sites.[67]

In United States, patients with Medicaid/Medicare had shorter survival after a diagnosis of SCCHN when compared with patients with private insurance. [105] Comorbidity reduces the survival in HNC.[4;106] A study of 1371 HNC patients found that comorbidity was present in 36.4% of the patients. Cardiovascular-, respiratory-, gastrointestinal comorbidity and diabetes showed a significant relationship with short-term mortality, which was 5.7%. Comorbidity impacts overall survival of newly diagnosed patients with SCCHN.[48] In two studies of TC non-smokers had better survival.[71;91] In HNC high alcohol consumption is associated with a higher risk of recurrence.[152] Death from non-cancer causes (competing mortality) is an important event in HNC.[152] A study from the Netherlands of 479 patients with stage III to IV HNC included in prospective trials, showed that

the 5-year cumulative incidence of competing mortality was 19.6%. In multivariate analyses, competing mortality was associated with gender female, increasing age, increasing Charlson Comorbidity Index, decreasing body mass index and decreasing distance travelled to the treating centre.[123]

Biological prognostic factors

Exophytic growth of the primary tumor[3] and lingual involvement of the primary tumor have shown to give prognostic information in TC.[119] The importance of histopathological differentiation in prognosis is not clear. Bentzen and Bataini have showed that well-differentiated tumors are associated with lower tumor control.[13;14] On the other hand, poorly differentiated TC are followed by poor survival.[51;119] Underlying genetic abnormalities may explain the discordance between the clinical outcome and the TNM status or location.[1;96] DNA aneuploidy is found in TC and is associated with worse prognosis.[124] The possible prognostic implications of EGFR and VEGR have been discussed earlier in this introduction.

Treatment-related prognostic factors

The most important changes are summarized in Table 3 below.

Table 3. Some important improvement in the treatment of HNC

Year	Author	Treatment	Study	Stage	Improvement
1998	Overgaard[134]	Radiotherapy +/- nimoraxole	Randomized, n=414	I-IV	+ 16% local control
2000	Pignon[141]	Concomitant cisplatin	Metaanalysis	I-IV	+ 8% 5-year survival
2006	Bourhis[21]	Accelerated radiotherapy without total dose reduction	Metaanalysis	III-IV	+ 2% 5-year survival
2006	Bourhis[21]	Hyperfractionated radiotherapy	Metaanalysis	III-IV	+ 8% 5-year survival
2006	Bonner[20]	Radiotherapy +/- anti EGFR – Cetuximab	Randomized, n=414	III-IV	+ 20 months in median survival

AIMS OF THE STUDY

In this study we wanted to answer the following questions:

1. Are modified TNM-based classification systems better prognostic tools than the original TNM classification?

2. Can new prognostic indices including patient and treatment variables based on multivariate survival analyses, give additional prognostic information compared to TNM alone?

3. What is the prevalence of HPV subtypes in TC in Norwegian patients, and has it changed over the past decades?

4. How does the presence of HPV correlate to clinical parameters, other markers and patient prognosis?

5. Dysfunction of spindle checkpoint proteins may induce chromosomal instability/aneuploidy. What is the level of expression of the spindle checkpoint proteins Mad2 and BubR1 in TC?

6. Can the expression of spindle checkpoint proteins serve as useful prognostic factors in TC?

PATIENTS

Series 1 - Prognostic indices, n=310

From 1960 until 1996 310 consecutively untreated patients with SCC of the tonsillar region without metastatic disease were admitted to the Norwegian Radium Hospital and/or the Department of Otolaryngology, the National Hospital in Oslo. The tumors were retrospectively staged according to the 4th edition (1978) of the TNM system.[75] Mean age of patients was 63 years and the male/female ratio 4/1. Most patients presented with advanced disease at the time of diagnosis, 59% had lesions staged T3-T4 and 65% had regional (N1-N3) metastases.

Tumor tissue was fixed in formalin and embedded in paraffin. We were able to obtain adequate tumor tissue from original archival tissue blocks of biopsies and surgical specimens from 199 of the 310 patients. Tissue sections were cut at 4 µm thickness and stained with hematoxylin-eosin and reevaluated by an experienced pathologist (Prof. Ole Petter Fraas Clausen). The original diagnosis of SCC was confirmed for all patients.

Complete follow-up and accurate cause of death were obtained by direct patient knowledge, review of outpatient and hospital charts, autopsy findings, direct contact with local hospitals, family physicians or in some cases by next of kin. The mean follow-up was 40 months. At the time of survival analyses 28% (87/310) patients were still alive. In the survival analyses event was defined as death due to the tonsillar carcinoma (47%), fatal complications during treatment (1%) or deaths of unknown causes (13%). Patients dying of other diseases 7% (23/310) or other head and neck cancers 4% (12/310) were treated as censored observations (under risk until death). Patient treatments are summarized in Table 4.

Table 4. Treatment in 310 patients with TC 1960-1996

Treatment		n=310	%
Surgery	resection primary tumor	87	28
	neck dissection	97	32
Radiotherapy	No	8	3
	< 50 Gy	46	15
	50 - 59 Gy	46	15
	50 - 69 Gy	78	25
	70+ Gy	130	42
Chemotherapy	No	237	76
	Yes[1]	73	23

[1] 16 patients received cisplatin-based chemotherapy (not concomitant) 1983-1996.

Series 2 - HPV, n=137

Adequate tumor tissue from original archival tissue blocks of biopsies and surgical specimens was obtained for 199 of the 310 patients. It was possible to isolate good quality DNA from 137 of the 199 tissue specimens, which defined the study population.

Series 3 - The spindle checkpoint proteins, n=105

From patients in series 2 we were able to measure spindle checkpoint proteins in 105 patients from which there was sufficient tumor tissue left in the paraffin blocks.

Patient selection and variation in therapy

Patient selection can represent an important bias in studies of markers and prognosis. This study was designed to reduce the patient selection to a minimum as all the consecutive patients of the two cooperating hospitals were reviewed. Both hospitals' files were checked, thus the chance of missing some patients was very unlikely. Sixteen patients were excluded from further evaluations. Eleven of them received no treatment because of either poor general condition or treatment refusal. Five

patients had disseminated disease at the time of diagnosis. With minimal selection within the hospitals we included 310 M0 patients who were treated with the intention to cure, which represent the world's second largest series of TC. In the same period 811 patients with ICD 145 were recorded in the Norwegian Cancer Registry, implying that our hospital series constituted about 40% of the whole country. Adjusted for the population our hospitals served, this indicates no selection of importance on the national level.

Table 5. Age, gender and stages in the two period

Variables	**Period**					
	1960-84 n=148			**1985-96** n=162		
Agegroup	**n**	**%**	**95% CI**	**n**	**%**	**95% CI**
< 60	44	30	23-38	79	49	41-56
60+	104	70	62-77	83	51	44-59
Gender						
Males	112	76	68-82	120	74	67-83
Females	36	24	18-32	42	26	20-33
Stage						
I	5	3	1-8	9	6	3-10
II	16	11	7-17	22	14	9-20
III	49	33	26-41	36	22	16-29
IV	78	53	45-61	95	58	51-66

In order to obtain a large patient series and thus a higher statistical power, the inclusion period was as long as 37 years, from 1960 to1996. Due to this long period, heterogeneity in staging and treatment can be confounding variables. New diagnostic modalities can result in stage "migration", which means that patients could have been classified into higher stages after for example computer tomography came into use. Computer tomography made it easier to visualize the extension of the primary and secondary tumors and to detect regional metastases earlier and have systematically been used since 1985.

However, the stage distribution did not change significantly for the two time-periods before and after 1985 (Table 5), and thus the use of computer tomography scans does not seem to have contributed to important stage migration. Table 5 also shows that the patients were significantly younger in the last period. Furthermore, a uniform use of protocols, procedures and close cooperation between the two hospitals should hopefully have reduced stage migration to a minimum.

The patients did not receive a uniform treatment, and we have explored this consideration in several ways. For example, chemotherapy was a significant variable in the univariate survival analyses, but not in the multivariate analyses. Even when chemotherapy was "forced into" a Cox model, the regression coefficients for the other variables were not notably changed, which indicates that there was no prognostic implication associated with the use of chemotherapy. This is supported by a multicenter study of Lewin and co-workers including 460 patients with HNC.[111] They found no survival benefits for patients treated with neoadjuvant chemotherapy.

Surgical parameters were not among the significant prognostic variables in this study. Lymph node dissection performed for regional metastases was tested and did not influence the survival. This may be due to the fact that the patients with regional metastases received adequate preoperative radiation therapy (no residual tumor tissue was histologically detected in 57% of the patients) and that the number of patients with advanced regional disease were relatively few. Local surgery was performed for diagnostic and therapeutic purposes, but this parameter was not tested in the survival analyses.

The two most significant treatment factors in this study were the total dose and the duration of radiotherapy. The standard total radiation dose since the 1980's has been 70 Gy and we selected 70 Gy and 50 days as cut-offs. We adjusted for these two variables in Paper 1 by including them in the prognostic indices derived and tested. In Paper 2 and 3 total dose and duration of radiotherapy were tested in the multivariate analyses together with HPV and BubR1, but did not emerge as significant factors. Thus the reported prognostic importance of HPV and BubR1 should not have been influenced by differences in radiotherapy regimens.

METHODS AND METHOLOGICAL CONSIDERATIONS

Tissue sampling and preparation

In this retrospective study fresh frozen material was not available and the analyses had to be performed on paraffin embedded tissue blocks. A recent study of formalin fixed cervical specimens back to 1931, showed that high quality DNA was extracted and successfully used for detection of HPV and sequencing.[17] We were able to obtain adequate material from original tissue blocks from 199 of the 310 patients. However, a previous study of telomerase expression consumed some of these tissue blocks.

In Table 6 below the distribution of some variables (age, gender, stage and period) for these 199 patients compared to the other 111 patients are shown. The 95% confidence intervals of the percentages did overlap, indicating no selection of importance. Similarly, no selection bias was found for the 137 patients where DNA was isolated when comparing with the other 173 patients without isolated DNA (data not shown). Also for the 105 patients included in the spindle protein study, no selection bias was found for age, gender, stage or period (data not shown).

Table 6. The distribution of some variables for 199 patients with tissue available compared to the other 111 patients

Variables	Tissue available					
	No n=111			Yes n=199		
Agegroup	n	%	95% CI	n	%	95% CI
< 60	42	38	29-48	81	41	34-45
60+	69	62	52-71	118	59	52-66
Gender						
Males	81	73	64-81	151	76	69-82
Females	30	27	19-36	48	24	18-31
Stage						
I	1	1	0-5	13	7	4-11
II	16	14	8-22	22	11	7-16
III	32	29	21-38	53	27	21-33
IV	62	56	46-65	111	56	49-63
Period						
1960-84	58	52	43-61	90	45	38-51
1985-96	53	48	39-57	109	55	48-62

Tissue microarrays

The use of whole sections of paraffin-embedded tissues is time-consuming in studies including many tissue sections. The tissue microarrays technique (TMA) for examining a large number of histological sections simultaneously was established in 1998.[101] Core needle biopsies from paraffin-embedded tissue blocks from many patients are arrayed in one new paraffin block. The analyses of one or a few master slides make it possible to study the entire cohort of tissue blocks from many different patients.

Our study included many specimens, but whole sections were used for Ki-67 and p53 analyses since TMA was not established in our laboratory at the time these analyses were performed. BubR1 and Mad2 analyses were performed later when the TMA technique was

established.[27] The most representative tumor areas were marked on the tissue blocks prior to tissue core sampling. Two or three tissue cores each 1,0 mm in diameter were sampled from these areas.

The advantages of TMA are that it is time-saving, many specimens are processed under the same conditions, the amount of archival tissue required is less and the costs are reduced due to lower amount of antibodies needed. A possible pitfall utilizing the TMA technique is that tissue cores are not representative of a tumor originally many cm in diameter, and may not represent the true distribution of protein expression within the tumor. However, the TMA has been compared to whole sections in several studies, and the validation showed good correlation between the two methods.[88] In a study of breast carcinomas antigen expression analyzed by immunohistochemistry (IHC) in 2-10 tissue cores were compared with antigen expression in whole sections, revealing that two tissue cores were comparable to a whole section in 95% of cases.[30] However, the representativity of antigens in tissue cores depends on the distribution of the antigen expression in a tissue. In our experience the uneven distribution of p53 in a tissue, makes tissue cores less suitable for p53 analysis and we therefore examined p53 in whole tissue sections. The distribution of BubR1 and Mad2 was relatively even so we assumed that 2-3 tissue cores from an area of representative tumor tissue gave representative results. Tissue loss or damage is another disadvantage of the TMA method. Old tissue blocks have a tendency to get fragile producing cracks, but this did not represent a major problem in our TMA sampling.

Changes in fixation protocols in the two periods and possible implications

There was a change in fixation protocols in the study period from unbuffered to buffered formaldehyde. These changes may have altered the detection of protein expression and the quality of DNA. The most important molecular changes induced by formaldehyde are the formation of hydroxymetylene bridges between proteins and between proteins and nucleic acids. These bridges mask the antigenic epitopes by altering the structure of the proteins.

In the HPV analyses we did not examine antigens, but there is a possibility that changes in fixation protocols have influenced the results. The change from unbuffered to buffered formaldehyde occurred at different times in the different laboratories. We examined tissue blocks from many pathology departments and have no information about when they changed the fixation technique. Due to this lack of information it is impossible to examine the effect of the different fixation protocols in this study.

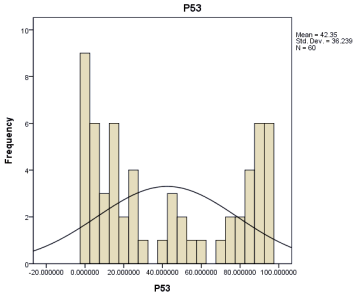
When we analyzed the distributions of p53, Ki-67, BubR1 and Mad2 in the two time periods, we found a modest left-sided displacement of the distributions (histograms), and lower mean and median in the oldest period only for Ki-67. However, the patterns (histograms) are much the same in both periods. Because of these similarities in distribution patterns and minor differences in mean and median values, we consider material from both time periods to be suitable for immunohistochemical analysis with the chosen antibodies.

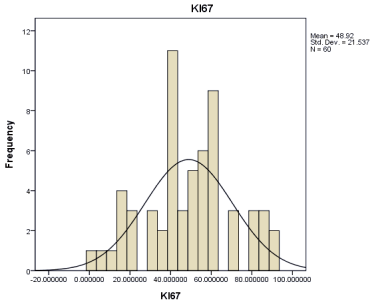
Table 7. Mean and median values p53, Ki-67, BubR1 and Mad in the two periods

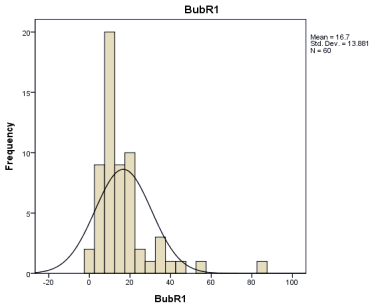
Variable	1960-84 n=60		1985-96 n=44-45	
	Mean	**Median**	**Mean**	**Median**
p53	42	29	45	41
Ki67	49	50	63 (p=0.001)	63 (p=0.001)
BubR1	17	12	20	18
Mad2	29	27	33	28

The distributions for the two periods are shown on the next page.

Figure 8 a: Some parameters 1960-84:







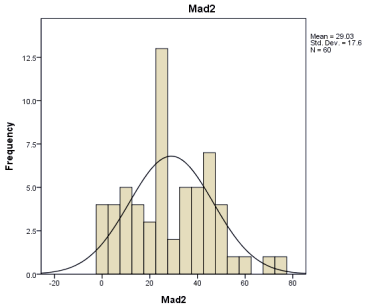
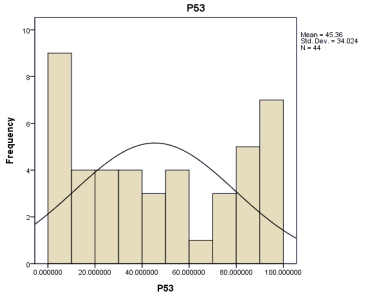
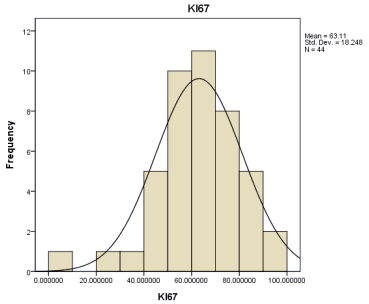
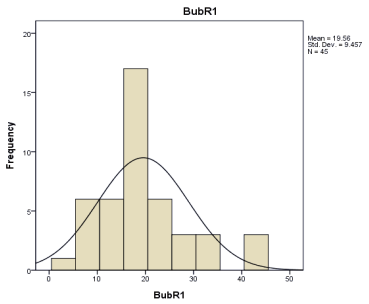
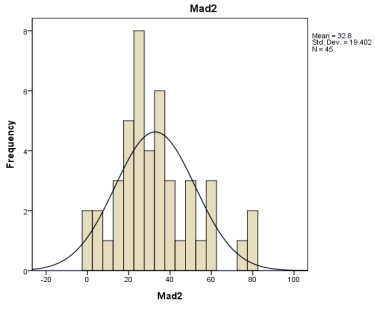


Figure 8 b: Some parameters 1985-96:









Immunohistochemistry of BubR1, Mad2, Ki-67 and p53

IHC refers to the process of localizing antigens (e.g. proteins) in cells of a tissue section by using antibodies binding specifically to epitopes in biological tissues. It is widely used in the pathology for diagnostic and prognostic purposes and in basic research to study the distribution and localization of specific proteins.

There are two strategies for IHC detection of antigens in tissues, a direct method and an indirect method. We utilized the indirect method, which is a two step procedure that had been established earlier in our group.[26] In the first layer an unlabeled primary antibody reacts with the tissue antigen and in the second layer the labelled secondary antibody reacts with the primary antibody. The second antibody coupled with streptavidin-horseradish peroxidase reacts with diaminobenzidine producing a brown staining where the primary and secondary antibodies are attached. Antigen detection can be significantly improved by treatment with an antigen retrieval e.g. citrate buffer, Tris-EDTA buffer and microwave treatment. Protein cross-links formed by formalin fixation may hide antigenic sites and are broken by antigen retrieval agents.

The protocols and antibodies against BubR1 and Mad2 used in Paper 3 have been described previously.[26] The specificity of the antibodies used is documented in previous publications and Western analyses verified that lysates from tumors showed protein bands at molecular weights consistent with those previously reported for these proteins.[26] The expression of Mad2 was found in the nucleus and BubR1 in the cytoplasma in normal tissue.[26] In tumor tissue there is some additional expression of Mad2 in the cytoplasm and some additional expression of BubR1 in the nucleus. The percentage of double expression was very low and was not registered. In this study positivity for Mad2 refers to the nucleus and positivity for BubR1 refers to the cytoplasm.

The IHC for Ki-67 and p53 detection has been described in Paper 2. The Ki-67 antibody was a pool of two mouse monoclonal antibodies (MIB1 and MIB3 mixed supernatants, 1:50 dilution) kindly provided by Dr J Gerdes, Germany. The p53 antibody used was a mouse monoclonal antibody BP.53-12, 1:100 dilution, Novocastra Laboratories Ltd., Newcastle upon Tyne, UK). The same staining procedure was used for both antibodies. Three hundred tumor cell nuclei were

counted in randomly selected areas from whole tumor sections. The nuclei were scored as either positive or negative and the percentage of positive tumor cell nuclei calculated. TMA for Ki-67 and p53 analyses would therefore not give additional information to the results already obtained.

False positive and negative results are always to be considered when using immunohistochemical techniques. It is therefore necessary to validate the antibodies used, either by Western blot analysis to see if they precipitate with protein bands of the expected molecular weight, or to use more than one antibody that shows similar results. The validity of the BubR1 and Mad2 antibodies has been documented in previous publications from our group,[26] and the validity of the Ki-67 and p53 antibodies has been documented in many previous publications from our and many other groups. We always use positive and negative controls for staining.

The Ki-67 antigen was originally identified in the early 1980s, by use of a mouse monoclonal antibody against a nuclear antigen from a Hodgkin's lymphoma-derived cell line. This protein was named after the researchers' location, Ki for Kiel University, Germany, with the 67 label referring to the clone number on the plate.[62] The Ki-67 antigen is encoded by a gene on chromosome 10. Ki-67 expression varies throughout the different phases of the cell cycle. Cells express the antigen during G1, S, G2, and M phases, but not during the resting phase G0. Ki-67 levels are low in G1 and S phases and rise to their peak levels in mitosis. Later in the mitotic phase (anaphase and telophase), a sharp decrease in Ki-67 levels occurs. Thus Ki-67 can be a reliable index of cellular proliferation. Ki-67 expression is related to prognosis in some cancers, such as breast-,[180] lung,-[162] bladder-[179] and laryngeal cancer.[154]

p53 plays a sentinel role in the pathways that prevent development of cancer by inducing apoptosis, DNA repair and cell-cycle arrest in response to different types of cellular stress. The majority of head and neck tumors harbour mutations affecting the TP53 gene. Loss of function of p53 is common in human cancers, and inactivation of the TP53 gene via mutations during tumorigenesis may result in inappropriate progression through the cell cycle after DNA damage, thus resulting in survival of cells that otherwise might have been destined to die. Accumulation of p53 which most often results from mutations, is associated with metastatic disease or poor prognosis in several cancer types, such as HNC,[166] oral carcinomas,[157] lung cancer,[44] colorectal cancer[136] and breast cancer.[12]

Polymerase chain reaction, sequencing and blasting

HPV is a single circular double stranded genome of ca 8 000 base pairs. In all the different HPV types the genome is organized similarly. It encodes for 6 early (E) and two late (L) genes. The transcription of viral DNA is regulated by early phase genes, while the capsid proteins involved in the viral spread, are regulated by late phase genes. E1-E2 proteins are required for DNA replication. E4-E5 proteins are needed for amplification of the viral genome. E6-E7 proteins of high-risk HPV induce cellular immortalization by regulating the functions of p53, p31 and pRb, pivotal proteins involved in apoptosis, DNA repair and cell cycle control.

Polymerase chain reaction (PCR) used for HPV detection serves as a technique to amplify a single or a few copies of a DNA sequence, thus generating thousands to millions of copies. The HPV detection protocol has been described in detail in Paper 2. Short; the first step was to confirm the presence and quality of DNA by targeting β-globin as a constitutive control, using PCO3/PCO4 as specific primers. Following amplification on a PCR cycler, a process where cycles of repeated heating (denaturation) and cooling (renaturation), enable the DNA polymerase to copy the target DNA. For evaluation, the products were run on an agarose gel alongside a DNA ladder, a molecular weight marker containing DNA fragments of known size. The size of the bands (or fragments) indicating the specificity of the PCR products are determined relative to the DNA ladder. Samples with representative bands indicative of good quality DNA were subjected to further (HPV) analyses.

General HPV detection was performed by using the universal HPV primers GP5+ and GP6+ and using a touchdown PCR protocol. Touchdown PCR is a variant of PCR that aims to reduce non-specific background by gradually lowering the temperature of the PCR cycles. A high initial annealing temperature specifies the targets for subsequent amplifications at lower temperatures. The resulting products were run on an agarose gel and positive bands indicated HPV DNA.

After excision from gel, the PCR products were purified using MicroSpin Columns S-300 HR (Amersham Pharmacia Biotech) according to the manufactures instructions, and then sequenced with ABI PRISM BigDye Terminator Cycle Sequencing and run on an ABI 3130 XL sequencer. DNA sequencing determines the order of the 4 nucleotide bases adenine, guanine, cytosine and thymine in a DNA molecule. It results in a succession of bases representing the primary structure of

a DNA molecule or a strand. The key principle is the use of dideoxynucleotides as DNA chain terminators. The 4 nucleotides are labelled with fluorescent dyes of different wave length and the data output is fluorescent peak trace chromatograms.

The results were analyzed using the NCBI Blast program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Basic Local Alignment Search Tool (BLAST) compares a query sequence with a database of sequences, identifying the origin (or source) of the sequence in question. The NBCI blasting program was utilized due to prior experience. In order to reduce the possibility of false positive and negative results the blasting process was repeated utilizing a second blasting program, Biology Workbench. The results from the two different blasting programs showed good correlation.

Many detection methods have been used to study the role of HPV virus including in situ hybridization (ISH), IHC, Southern Blotting and PCR. ISH technique employs the use of HPV type-specific radioactively labelled DNA oligonucleotides as probes complementary to various HPV DNA type sequences. This method is now considered the best method for HPV detection due to its high sensitivity and is the method of choice today. IHC have low sensitivity because it can only detect virus present in more than 10 copies of viral DNA pr cell. Southern blotting combines electrophoresis separated DNA fragments and fragment detection by probe hybridization, requiring a significant amount of DNA and is therefore less sensitive than PCR.[63]

We used PCR for HPV detection because PCR was a common method for HPV detection at the time this study was initiated enabling us to compare our results with other studies.[63;130;170] Other reasons were that PCR has a high sensitivity and our laboratory had good experience in utilizing PCR methods.[23]

However, several have called for more standardised approaches to HPV testing in HNC.[23;149] Different methods are used to detect HPV; HPV DNA, HPV RNA, HPV proteins and cellular proteins. These have variable analytic sensitivity and specificity, and may have given false positive and false negative diagnosis of HPV in oropharyngeal carcinomas. Some argue that detection of high risk HPV by consensus PCR alone seems to be insufficient to accurately classify tumors.[149] Studies of cervical carcinomas have indicated that any single method or technique for the detection of HPV may underestimate the true prevalence of HPV.[10] Two new diagnostic algorithms have

emerged. The first advocates screening for p16 by IHC followed by detection of HPV DNA by ISH. The second recommends detection of p16 followed by consensus PCR.[149;163]

General primers for HPV were used in this study because of their ability to identify many different human HPV types simultaneously and thereby reduce the time taken to perform the analyses. Specific primers require many PCR repetitions which might increase the risk of contamination. In designing pairs or multiple sets of primers to participate in the same reactions, complementarity should be avoided to reduce formation of primer-dimers. Primer-dimers reduce the availability of the specific primers to participate in the polymerase driven reactions and thereby reducing the efficiency in the amplification process. Excessive DNA fragments in the form of primer-dimers are easily distinguished from the HPV bands due to significant differences in molecular weight.

A disadvantage of the general primers is the relatively large size of the PCR fragment especially in samples that yield poorly amplifiable DNA as in formalin-fixed paraffin embedded material.[99] The sensitivity is also influenced by the temperature of the PCR cycles. High temperature decreases the sensitivity which might cause loss of some of the relevant HPV genotypes. The general primers have been modified over time and the primers in use today can detect a wider range of mucosal HPV compared to the primers applied in this study. This presents other challenges. It is not hard to contemplate that if a patient has multiple infections with various genotypes, these are amplified in an unpredictable way as the PCR reaction favours the targets being closest to the primers in complementarity being represented in the highest quantity. Further, if several genotypes are being amplified simultaneously, this will jeopardise the sequencing reaction because of the multiplicity of targets. We believe these considerations have influenced on our results concerning the detection degree and the HPV genotype representation of the investigated samples. This is supported by other studies. In a study of cervical cancer samples comparing the general primers GP5+/GP6+ with the MY09/MY11 primer set, the MY-PCR detected 14 of 30 (90%) samples with multiple HPV types, whereas the GP+PCR detected 14 of 30 (47%) samples with multiple HPV types.[144] In the same study each primer set amplified some HPV types better than others causing biases in sensitivity. GP+PCR detected fewer samples containing HPV types 52,53 and 61 compared to MY-PCR, but the predominant HPV types found in patients with cervical

cancer (HPV 16, 18, 32 and 45) was detected with equal frequency by both PCR systems. Another reason for reduced sensitivity towards certain HPV types can be the circulation of a variant with additional sequence mismatch over the primer binding site.[31]

The amplification potential of PCR makes it vulnerable for contamination by targeting spurious DNA products.[23] Contamination with extraneous DNA is addressed with laboratory protocols and procedures that separate pre-PCR mixtures from potential DNA contaminants. Our use of separate rooms for PCR-setups, careful handling of the tissue blocks utilizing a new sterile scalpel and new gloves for each block, disposable plastic-ware and thoroughly cleaning the work surface between reaction setups reduce contamination. Contamination was monitored running negative controls. False positive signals can also occur due to self-priming of DNA because fragmented DNA caused by formalin fixation can act as primers.[81]

The sequencing is today automated due to its great speed and dye terminator sequencing is the mainstay. The limitations of dye terminator sequencing include dye effect due to differences in in-cooperation of the dye labelled chain terminator into the DNA fragments, resulting in unequal peak heights and shapes in the chromatogram.[2]

Statistical analyses

Survival analyses take into account both the event recorded (relapse or death) and the time to the event. Observations which are censored (the event has not occurred at the time of the study analysis) are included in the risk calculations until time of censoring. The Kaplan-Meier method gives a good graphical presentation of a survival curve,[97] while the log rank test is a significance test of the differences between survival curves.[140] Clinical research often involves several variables with a need for relative adjustment of their impact.[85] Three main regression models are available. Multiple regression can be used for analyses of variations in a continuous variable (e.g. age), logistic regression for a binary response (event, no event) and Cox regression for survival (both event and time to event).

Calculating survival curves in univariate survival analyses and the significance testing of the differences between the survival plots have few pitfalls. The most important concern is the problem of repeated analyses.[155] We solved this in two ways in univariate survival analyses. First, we tested earlier reported thresholds if relevant. Second, we divided continuous variables such as Ki-67 into quartiles by using the 25%, 50% and 75% percentiles as cut-off levels, and thus did not search for "suitable" thresholds. Another advantage utilizing quartiles is the ability to identify U-effects (if high or low values of a continuous variable are associated with bad prognosis). p53 was not used as a continuous variable in survival analysis. The co-authors have previously used 5% cut off for colon cancer which correlated with the mutations present. We assumed it was suitable to have a higher cut off for ENT cancer since publications at that time indicated less accordance with p53 accumulation and mutations in ENT cancers. We also assumed that a few tumors with <10% positivity had mutations. Thus we chose 10% as a threshold since this value was commonly used by others. The median as a threshold value for BubR1 expression has previously been evaluated by Burum Auensen et al.[28] Screening by quartiles did not show that other threshold values were more relevant than the median of BubR1 expression.

The form of the candidate variables and the procedures used in stepwise testing in Cox regression can greatly influence which variables will remain in then "final model" ("significant" or "not significant"). Thus, a shortcoming of modern survival regression is that their extended use

implies a risk of introducing false negative as well as false positive prognostic factors in the medical literature.[82] One way to solve this when the patient series is large enough, is to both derive and validate new prognostic indices in the same patient series as we did in the first paper. The problem of many repeated analyses in the Cox-analyses was also handled by a simplified approach (few analyses) where e.g. the focused factor (HPV or BubR1 expression) was tested against the established variables (TNM, age and gender).

The prognostic indices were estimated by multiplying the regression coefficient with the group value associated with adverse survival for each variable. The higher the index, the worse was the survival. In the first paper we divided the prognostic scores into three groups: low-risk (the lowest 25% of the index-distribution), medium risk (2^{nd} and 3^{rd} quartiles) and high risk (the highest 25% of the index-distribution). To make calculation of prognostic indices as simple as possible, we recoded all variables so the low risk values (i.e. females, HPV-positive, age < 60, high BubR1 expression) were the reference group, coded as 0.

The Cox regression model is based one main assumption, the proportional hazards (death risks).[53;173] The model assumes that the relative death risk or hazard ratio (HR) for patients in for example two age groups (< 60 versus ≥ 60) is the same (constant) at different times from start of the observation. We tested this assumption with plots. In Paper 3 this violation was visually so large, that we recoded TNM stage I-II to be the reference group (coded 0). This was in contrast to the other papers where TNM stage I was the reference group and stage II-III were grouped together.

SUMMARY OF RESULTS

Paper 1: Different prognostic indices in 310 patients with TC

Several modifications of the TNM system have been reported to be better prognostic tools than the original classification in HNC, but none of these modifications had been tested in a large series of TC.

The 5 reported TNM-based stage modifications were all highly significant predictors of survival in our 310 patients. The patients were randomly divided into a derivation group (n=199) and a validation group (n=111). In univariate survival analyses of the derivation group, 7 patient- and treatment variables were significant prognostic factors ($p < 0.05$). In multivariate survival analyses of the derivation group, 4 host- and treatment variables that indicated shorter disease specific survival were identified; age ≥ 60, gender male, total radiation dose < 70 Gy and duration of radiotherapy > 50 days. We then created two sets of prognostic indices based on pretreatment data (TNM + age group + gender) and a treatment index (TNM + age group + gender + radiation dose + duration of radiotherapy).

When applying these indices to the validation group, all 4 indices separated the patients into different risk groups of high statistical significance ($p < 0.001$). By adding the two host variables or the two treatment variables, the prognostication improved for TNM. The TNM stages were distributed in the different risk groups based on the pretreatment index. For example, of 63 patients in TNM stage IV, 6 came in low risk-, 18 in medium risk- and 39 were in high-risk groups. By adding the treatment variables into the prognostic indices, the differences between the survival curves increased, indicating an even better prognostication and a further shift in distribution between the risk groups were noticed.

Paper 2: Human papillomavirus (HPV)-positive TC are frequent and have a favourable prognosis in males in Norway

The objective of this study was to determine the prevalence of HPV subtypes in TC in Norwegian patients, and to correlate the presence of HPV to clinical parameters and prognosis. Furthermore, we wanted to explore the significance of Ki-67 and p53 positivity and their individual relationships to HPV.

Seventy-one (52%) of these specimens were HPV-positive, 56 males and 15 females. Age was significantly associated with HPV-positivity. Of patients younger than 60 years, 62% were HPV-positive compared to 43% of patients 60 years or older ($p<0.05$). HPV-positivity was significantly more frequent (64%) in the latter period 1985-1996 of the patient series compared to 38% in the first period 1960-1984 ($p<0.01$). p53 positivity was recorded in 71% of the patients. The distribution of HPV-positivity versus negativity was not statistically significantly different for gender, stage, T- and N categories, p53 positivity or Ki-67 expression. The HPV-16 subtype was most predominant, found in 62 of 71 (87%) positive tumors.

HPV-positive patients survived longer than HPV-negative patients ($p< 0.05$), with a 5-year survival of 54% for HPV-positive versus 33% for HPV-negative tumors. When stratifying the HPV survival analyses for gender, a more favourable survival was found only for HPV-positive men ($p<0.01$), whereas HPV-positive and HPV-negative females had overlapping survival curves. p53 positivity or high Ki-67 index did not correlate with survival. The unadjusted HR for HPV-negative patients (versus positive) was 1.6 (95% CI: 1.01-2.5).

In multivariate, stepwise analyses where HPV was tested against known prognostic factors, HPV-positivity was found to be a significant prognostic factor in addition to stage, age group and gender ($p < 0.05$). The HR for HPV-negative patients was 1.6 (95% CI: 1.01-2.6) when adjusting for period, stage, age group and gender.

Paper 3: Reduced expression of the spindle checkpoint protein BubR1 correlates with bad prognosis in TC

Spindle assembly checkpoint proteins such as Mad2 and BubR1 are important for normal mitosis. The aim of the present study was to examine their expression in TC, their possible impact on prognosis and correlation to clinical variables, the prevalence of HPV, p53 status and Ki-67 positivity in 105 patients.

BubR1 and Mad2 were both expressed in TC. The median numbers of positive tumor cells were 16% and 27%, respectively, for BubR1 and Mad2. BubR1 expression was mainly cytoplasmic, whereas Mad2 expression was mainly nuclear. No significant correlations were seen between BubR1 and Mad2 expression, and no significant relationships between each protein and clinical data, HPV status or p53 accumulation were found. HPV status was not correlated to levels of BubR1 expression. BubR1 expression was significantly correlated with Ki-67 positivity ($r=0.4$, $p<0.01$), whereas Mad2 expression was not.

A significantly reduced survival was found for patients with tumors having low expression of BubR1, with a 5-year survival of 25%, whereas a 5-year survival of 60% was seen for patients with tumors having high BubR1 expression ($p<0.01$). Analyzing the survival for BubR1 according to quartiles, did not reveal significant trends when comparing the 4 groups. BubR1 expression showed significantly different survival in age groups < 60 or ≥ 60, in males, in stage III and stage IV, in HPV-negative patients and in p53 positive patients. The unadjusted HR for low BubR1 expression was 2.2 (95% CI: 1.3-3.7).

In multivariate, stepwise analyses where BubR1 was tested against known prognostic factors, BubR1 expression was a significant prognostic factor in addition to stage, age group and HPV status ($p < 0.05$). The HR for patients with low BubR1 expression was 2.0 (95% CI: 1.1-3.7), adjusted for period, stage, age group, gender and HPV status.

DISCUSSION

Prognostic factors

The TNM system is primarily a staging system and it is regularly revised. These revisions are partly based on prognostic information from different sources. The main purpose of the TNM system is to have robust and reliable staging definitions over time. This makes it easier to describe and compare patient populations over decades, between countries and institutions and gives adequate frames in controlled studies.[83]

In order to develop a better assessment of prognosis at least two options exist. The TNM system can either be modified between the official international revisions, or we can maintain the current, virgin TNM classification and add new variables to it. Regression models such as the Cox regression model designed for time-to-event analyses have for a long time been available in user-friendly statistical software. The regression models give regression coefficients which are estimates for the relative death risk. These can as shown in Paper 1 and 2, be used relatively easily to develop practical prognostic indices for clinicians.

In Paper 1 we showed that the TNM stages and the 5 earlier reported TNM-based stage modifications were all highly significant prognostic factors in this large series of TC. Although two of the TNM modifications seemed to be better, it was not possible to declare a sure winner compared to the virgin TNM. However, a simple pretreatment prognostic index which added gender and age to the TNM classification defined especially high-risk patients very well (Figure 2a, Paper 1). In order to intensify the treatment and follow-up for high-risk patients, tools to identify these patients with high precision are mandatory. Tests and reports of modifications of the TNM system are useful inputs to the next revision of the TNM. However, with the aim to predict prognosis even more optimally, our data and indices support the strategy to add new and perhaps simple prognostic factors to the virgin TNM.[83]

We identified 4 important prognostic factors: age, gender, total radiation dose and duration of radiotherapy. A separation in pretreatment and treatment-based indices gave complementary

information about the risk groups. We believe it may be useful to separate indices based on pretreatment information and indices based on information available after the initial therapy has been completed. The treatment based factors are here referred to as prognostic factors, in contrast to predictive factors which describe the responsiveness of a particular tumor to a specific treatment and determine which treatment is best.[116] These indices may be useful for clinicians by giving information about the need for additional therapy. The two most significant treatment factors in this study were the total dose and the duration of radiotherapy. Our finding of the importance of a total dose of 70 Gy is in accordance with current international treatment programs.[127] The implication of shorter duration of radiotherapy is now realized in accelerated or hyperfractionated radiotherapy regimens.[21] Bentzen and Thames also found treatment time to be of prognostic value.[14;172]

Many papers have presented different possibilities to regroup the staging system in order to obtain more homogenous stages and to yield better prognostic information.[117] One of the criticisms against the established TNM classification for HNC has been that stage IV includes too many patients compared to the other stages. Our data showed that the distribution of patients between the different stages is more even in the reported stage modifications compared to the original TNM system. On the other hand, the TNM stage system is well established internationally, and in our opinion TNM should only be changed if it results in a major prognostic improvement which cannot be achieved with other adjustments.

In Paper 3 we found an association between low BubR1 expression and poor prognosis. This implies that we have generated a new hypothesis regarding prognosis which has to be validated in other series. However, if we are able to collect large patient series as we did in Paper 1, we can divide the material randomly into two groups, a derivation group and a validation group and present validated indices. This approach has several advantages. It is less time consuming and confounding factors may be reduced since it uses the same patient series, the same data collection and the same management.

An alternative had been to include period in the multivariate analyses in all three papers. In Paper 1 it was not done as the main purpose was to derive and validate a prognostic index using standard variables and to validate different TNM classifications with our data. Furthermore, the regression analyses in Paper 2 and 3 were based on the findings from Paper 1 (the pretreatment

variables TNM, age and gender. I Paper 2 HPV-negative patients had an unadjusted HR of 1.6. When period (1960-84 versus 1985-96) also was included and adjusted for, HPV status obtained only a minor change in HR to 1.5, while period had a HR of 1.6. The HR for HPV-negative patients was 1.6 when adjusting for period, stage, age group and gender. In Paper 3 patients with low BubR1 expression had unadjusted HR of 2.2. The HR for BubR1 remained high, 1.8 adjusted for period, the latter had a HR of 2.0. BubR1 remained significant with a HR of 2.0 after adjusting for period, stage, age, gender and HPV. This argue for a conclusion that the oldest period did not give significant noise in the data and analyses, and thus did not challenge the main findings that HPV and BubR1 were important prognostic factors in our study.

HPV

In our study 52% of patients with TC tested for HPV showed HPV-positive tumors. Our prevalence of 52% is similar to the value in a review of 432 patients comprising 27 series where 51% of TCs were HPV-positive.[170] The HPV-16 subtype was predominant, found in 87% of positive tumors. It is well documented that HVP-16 is the most frequent subtype.[170]

When we combine our findings with the data from the Norwegian Cancer Registry shown earlier in this thesis, we can summarize 5 important and related observations. 1: The incidence of tonsillar cancer in Norway has increased from 1960, especially in men. 2: The prevalence of HPV-positive patients has increased in the period from 1960 to 1996. 3: HPV-positive patients have a lower age at diagnosis. 4: There is a marked improvement in relative survival in tonsillar cancer in Norway from 1960 to 2006, mostly for males and for patients < 60 years. 5: HPV-positive, male patients have a more favourable prognosis.

HPV-positivity was significantly more frequent (64%) in the latter period 1985-1996 of the patient series compared to 38% in the first period 1960-1985 ($p<0.01$). In the county of Stockholm, the proportion of HPV-positive TC increased significantly both from 1970 to 2000 ($p < 0.0001$) as well from 2000 to 2007 ($p < 0.01$), with 68% HPV-positive cases in 2000-2002, 77% in 2003-2005 and 93% in the period 2006-2007. The prevalence of HPV-positive tumors almost doubled within each decade between 1970 and 2007, in parallel with a decline of HPV-negative tumors. The

prevalence of HPV-positive cancers is still increasing in Stockholm, suggesting an epidemic of a virus-induced TC.[130] Also in Greek patients with TC a tendency towards an increase in the proportion of HPV-positive tumors has been reported when comparing the percentage of HPV-positive tumors collected between 1992-1998 with those collected between 2000-2007.[150] These different findings are summarized below.

Table 8. The prevalence of HPV-positive TC in some countries over decades

Country	Period	Total n	HPV %	95% CI
Norway	1960-1984	63	38	26 – 51
Norway	1985-1996	74	64	52 – 74
Greece	1996-2007	28	43	24 – 63
Germany	1996-2005	29	76	56 – 90
Sweden	2000-2002	47	68	53 – 81
Sweden	2003-2005	52	77	63 – 87
Sweden	2006-2007	46	93	82 – 99

Thus, TC may represent medical history. Which other neoplasm has over a few decades (30-40 years) nearly completely changed its etiology? Identical HPV-16 DNA has been found within three couples where the husband and wife developed TC within 12 months of each other, revealing the potential infectious nature of oropharyngeal cancer.[6;69] There is strong evidence that this rapid increase in HPV-positive TC is due to changes in sexual behaviour.[66] Husbands of patients with cervical cancer have a higher risk of TC.[80] Sexual partners of patients with HPV infection develop a higher risk of HNC.[132] An increased HPV risk of oropharyngeal cancer is found in patients with a high lifetime number of heterosexual partners, young age at first intercourse and a history of orogenital sex.[165] In a hospital-based, case-control study of 100 patients with newly diagnosed oropharyngeal cancer and 200 control patients without cancer, a high lifetime number of vaginal-sex partners (26 or more) was associated with oropharyngeal cancer (OR 3.1), as was a high lifetime number of oral-sex partners (6 or more) (OR 3.4). The degree of association increased with the number of vaginal-sex and oral-sex partners.[45] A support for a possible transmission of genital HPV to fingers have been found in a study from Sweden. Of 13 patients with a history of both cervical

and finger SCC, HPV-16 was found in 5 finger SCC in 7 patients with HPV-16 cervical SCC.[58] Based on a simple anatomical logic and the above findings, we propose that the increase in oral sex with subsequent HPV infection is the most important change in the risk picture explaining the increase in the TC incidence.

In a meta-analysis of the effect of HPV status, the overall and disease free survival were significantly improved for patients with HPV-positive tumours. While the majority of studies (21 studies) reported an improved prognosis, several studies reported no difference (9 studies) or worse outcome (3 studies). For these three groups of studies, evaluation of HPV prevalence by tumour site may have provided an explanation for the reported differences in outcome. Studies reporting a worse prognosis or no difference in outcome had a much higher prevalence of HPV-positive tumours overall compared to those reporting a favourable prognosis. One explanation for the high prevalence of HPV infection reported in these studies may be that the majority of the HPV-positive tumours carried either low-risk or uncharacterized HPV types.[145] Further evidence for HPV as a strong and independent prognostic factor was recently reported in a retrospective analysis of a randomized trial with 721 patients with stage III and IV oropharyngeal carcinomas. The patients were classified in low, intermediate, or high risk groups regarding survival based on four factors: HPV status, pack-years of tobacco smoking, tumor stage and nodal stage.[7]

Most reports of HPV as a favourable prognostic factor have not discussed a possible gender difference. In 2003 Ritchie reported an association between gender and HPV status with respect to survival in 139 patients with carcinomas in the oral cavity and oropharynx. It was reported that HPV-positive males had better prognosis than HPV-negative males, whereas a similar difference was not found in females.[147] However, only 8 females of the 139 patients in the study of Ritchie were HPV-positive, and the authors warned about generalizations of this possible gender difference due to low numbers. The study of Ritchie and ours are among the largest patient series presented with HPV analyses in TC, and indicate that other studies with less patients may not have detected this gender difference due to lack of statistical power. In our first study of all 310 patients, gender gave additional prognostic information to TNM stage and age. As our multivariate analyses also found both gender and HPV status to be independent predictors together with stage and age, there seems to be both a gender and a HPV effect on survival. As HPV vaccination may be a future tool to reduce the incidence of TC, later studies should carefully examine this possible gender difference

regarding the impact of HPV presence on prognosis. By pooling and reanalyzing the world's 3-4 largest published studies, the HPV and gender interaction in TC should be clarified.

Why HPV-positive TC in general has a better clinical outcome than HPV-negative tumors is not known. There are several hypotheses to explain this difference. It may be related to better treatment effects of radiation and chemotherapy due to intact apoptotic capacity and to better immune response against HPV.[54] HPV-positive cells may suffer from hypoxia and can be more easily induced to apoptosis. The genome of HPV-positive cancer cells may be less unstable. [50;171] Another possibility is that a different signalling pathway may be affected associated with less aggressive behaviour. It is reasonable to assume that deregulation of relevant cellular signalling pathways differ between HPV-positive and HPV-negative TC, respectively, and that the deregulation of growth is associated with more aggressiveness in HPV-negative tumors[109].

An impact of the immune response on tumor development is supported by the report of increased prevalence of TC in patients with impaired immune systems. Our study is consistent with the hypothesis that therapeutic anti HPV-16 vaccines developed for cervical cancer may also be of benefit in the management of TC.[46] As the mean time from HPV infection to cancer development is about 12 years, the prevalence of HPV induced TC will increase steadily in years to come. Thus prevention of this cancer can be both by vaccination programs in young age and education about the risk of oral sex and safer sexual behaviour. In addition, two questions should be answered: What information should be given and which actions should be taken in males where the female partner has received the diagnosis of a HPV-positive cervical cancer? And the opposite relation; what information should be given and which actions should be taken in women where the male partner has received the diagnosis of a HPV-positive TC?

Shuman has given some specific recommendations as follows:[160]

- *To ensure that patients receive factual and accurate information, HPV should be discussed as a possible etiologic agent with all patients with oropharyngeal squamous cell carcinoma.*
- *Patient factors and physician judgment should dictate the utility of discussing HPV in the setting of HNC presenting at other head and neck sites in which HPV is unlikely to be a causative factor.*

- *The transmissibility of HPV may be discussed at the discretion of physicians, acknowledging that other than standard barrier methods, there exists no convincing evidence that specific behavior modifications or sexual contact precautions are necessary.*
- *The favorable prognosis of HPV-related neoplasms may be discussed with patients as is clinically appropriate, understanding that treatment is not currently adjusted based on this information, and other factors can significantly affect prognosis.*
- *Physicians may discuss the belief that HPV vaccination theoretically might help prevent certain HNC despite the absence of conclusive evidence.*

Fei et al explored the relationships between VEGF, EGFR, HPV, response to radiotherapy and clinical outcome in 85 TC. There was a significant inverse relationship between EGFR and HPV status. VEGF and EGFR were risk factors for local recurrence and disease-specific death in univariate analyses, but the associations weakened after adjustment for HPV. Among patients treated with radiotherapy, VEGF was associated with disease-specific death after adjusting for HPV and TNM stage. High-VEGF-expressing tumors positive for EGFR had a worse prognosis than all other groups combined after adjusting for HPV and TNM stage. However, HPV was a stronger prognostic marker than VEGF and EGFR in TC.[56]

The improved prognosis and treatment responses to chemotherapy and radiotherapy by HPV-positive tumors may suggest that HPV detection is required for better planning and to individualize patient treatment regimes.[66] In a prospective study the association of tumor HPV status with therapeutic response and survival among 96 patients with stage III or IV SCCHN of the oropharynx or larynx was evaluated.[55] All patients received two cycles of induction chemotherapy with intravenous paclitaxel and carboplatin followed by concomitant weekly intravenous paclitaxel and standard fractionation radiation therapy. HPV-positivity was found in 40% of the patients. Compared with patients with HPV-negative tumors, patients with HPV-positive tumors had higher response rates after induction chemotherapy (82% versus 55%, $p = 0.01$) and after chemoradiotherapy (84% versus 57%, $p = 0.007$). Patients with HPV-positive tumors had improved overall survival compared to those with HPV-negative tumors.[55] We suggest that a randomized trial should be performed to test the hypothesis that HPV-positive patients with TC can receive less intensive treatment without loss of quality or duration of life.[125] In Norway, routine detection of

HPV has not been done up to now due to the costs. HPV detection in TC should be included as a routine analysis according to recent guidelines in USA.[131]

p53 positivity is correlated with mutations of the TP53 gene, resulting in nuclear accumulation of dysfunctional p53 proteins with longer half-lives. Other mechanisms however, may lead to non-mutational nuclear accumulation and stabilization of the p53 protein.[42] Since the E6 protein of HPV16-18 binds and degrades p53 protein, the p53 negative tumors represent a rather heterogeneous group with respect to p53 function. p53 negative tumors in the present study thus presumably include those with wild type p53 as well as those with p53 inactivation by HPV and those with nonsense mutations. In one study HPV-16 DNA was detected in 72% of 100 paraffin-embedded tumor specimens, and 64% of the patients with cancer were seropositive for the HPV-16 oncoprotein E6, E7, or both.[45] Thus, the HPV-mediated p53 inactivation by E6 may be an early and transient event during tumorigenesis, with later reestablishment of p53 function. This may contribute to the contradictory results reported in the literature concerning associations between p53 immunoreactivity, prognosis and HPV status within these tumors.

p53 is a tumor suppressor protein important for cell cycle regulation. It is involved in apoptosis induction and is required for growth arrest following DNA damage. Ki-67 is a molecular marker of tumor growth fraction and of cellular proliferation. In a small study of 33 patients with oropharyngeal carcinomas (11 TC), HPV-positive tumors had higher Ki-67 and lower p53 staining scores compared with HPV-negative tumors.[52] We did not find any significant correlations between HPV status and Ki-67 or p53 positivity respectively, in TC. Two other studies of 42 and 30 patients with HNC did not find any correlation between HPV status and p53 positivity,[11;103] similar to the results from our study. Hafkamp et al also support our findings as they report no correlation between HPV status and Ki-67 and p53 expression.[71]

The spindle checkpoint proteins

The mechanisms of the spindle assembly checkpoint are still not completely understood. A recent publication showed that BubR1 may have at least three different functions.[90] The complexities warrant a more careful discussion and interpretation of our findings.

BubR1 and Mad2 were both expressed in TC. No significant correlations were seen between BubR1 and Mad2 expression, or between each protein and clinical data, HPV status or p53 accumulation. BubR1 expression was significantly correlated with Ki-67 positivity, whereas Mad2 expression was not. The induction of p53 by mitotic checkpoint activation seems to be essential for protecting cells against the abnormal chromosomal ploidization induced by mitotic checkpoint failure.[59] Furthermore, p53 activation in response to mitotic spindle damage requires signalling via BubR1 mediated phosphorylation.[68] This suggests a cross-talk between the mitotic checkpoint and p53. We did not find any correlations between the levels of BubR1 and Mad2 expressions respectively, and p53 accumulation. However, low BubR1 expression was a more apparent prognostic factor within patients with p53 positive TC. This suggests that p53 dysfunction as indicated by p53 accumulation is of importance for the poor prognosis of tumors with low BubR1 levels. This is consistent with the documented crosstalk between these two proteins for maintaining proper mitotic checkpoint function preventing CIN.

We have shown that BubR1 expression is a significant prognostic factor in TC. Low BubR1 expression was associated with poor survival and was a significant prognostic factor both in univariate- as well as in multivariate survival analyses. Stratified survival analyses of BubR1 revealed significantly different survival times within TNM stages III and IV, illustrating that BubR1 is a novel, robust prognostic marker in TC in addition to the existing ones.

Correlations between BubR1 expression and prognosis have to date not been reported for many cancer types. In a series of 104 patients with bladder carcinomas studied by Yamamato et al,[177] overexpression of BubR1 correlated with higher histological grade, tumor recurrence, disease progression and high cell proliferation. In a recent study of 117 resected pancreatic head adenocarcinomas, high BubR1 expression was shown to be an independent, adverse prognostic factor for survival.[64] In 160 patients with ovarian cancer high BubR1 expression was associated

with shorter recurrence-free survival.[108] A study of 70 oral SCC found that high BubR1 expression was associated with shorter survival and in contrast to our results, they also showed that HPV was more prevalent in samples with a high BubR1 expression.[115]

In a study of 181 cases with gastric cancer, high BubR1 expression correlated with DNA aneuploidy, advanced stage and poor prognosis. The authors transfected gastric cancer cell lines with BubR1 to observe the significance of the change in BubR1 expression. Enforced expression of BubR1 resulted in changes to the ploidy patterns and high proliferation activity as measured by Ki-67 expression. These clinical and in vitro data may indicate that high expression of BubR1 may be one causative factor for the induction of DNA aneuploidy and progression of gastric cancer.[5] Our results also show a significant correlation between BubR1 expression and Ki-67 positivity in TC and a similar association has been previously demonstrated in ulcerative colitis associated[27] as well as in sporadic colorectal cancers.[28]

Rizzardi et al studied BubR1 expression in 49 patients with oral SCC. Tumors with overexpression of BUBR1 were associated with a less advanced pathologic stage and showed a tendency of longer survival periods which supports our findings.[148] Thus so far our study is only one of two studies to find low BubR1 expression as a bad prognostic factor. This contrasting finding may have several explanations: The mechanisms behind BubR1 reduction and the ensuing interference with tumor growth may differ in various types of tissues and their respective neoplasms (SCC versus adenocarcinomas). In our material the expression of BubR1 seems to be higher than in other reported studies, which may indicate an increased disruption of BubR1 regulation. Furthermore, disruption of BubR1 function may be associated with increased as well as reduced amount of protein present.

Under normal conditions BubR1 participates in preventing premature advance from metaphase to anaphase during mitosis and is activated when the spindle microtubules are not correctly aligned with the chromosomes in metaphase. DNA ploidy in HNC has been studied in several patient series,[19] and TC have previously been shown to display a high frequency of aneuploidy.[124] In a report published by Hass and collaborators,[78] DNA aneuploidy was more frequently seen in advanced head and neck tumors and lymph node metastases from oral carcinomas mainly harboured aneuploid tumor clones.[79] In a patient series of 66 cases with TC a high degree of aneuploidy was found in most tumors. HPV- positive tumors had a lower degree of

aneuploidy than HPV-negative tumors with a non-significant trend of worse survival associated with aneuploidy.[124] These findings are consistent with an association between low BubR1 expression and aneuploidy also in TC, and may explain the poor prognosis in these patients.

A defective mitotic checkpoint has been proposed to contribute to chromosomal instability. In 39 clear-cell renal cancers and 36 normal kidneys, expression of spindle proteins were analyzed. Overexpression of BubR1 was significantly correlated with CIN and tumor grade. The authors concluded that BubR1 overexpression plays a role in cytogenetic and morphologic progression of clear-cell renal cancer.[143] In a series of 19 patients with invasive ductal breast carcinoma, the status of chromosomal and intrachromosomal instability and the expression for two genes involved in the mitotic spindle checkpoint pathway, Bub1B and Mad2L1, were examined. All breast cancers demonstrated higher chromosomal instability rates in tumor samples than in controls. Bub1B mRNA levels, but not Mad2L1 levels, correlated with intrachromosomal instability.[156]

Poor survival for patients with low BubR1 expression was more pronounced in HPV-negative patients, in p53 positive patients, in males and in TNM stage III-IV. However, in all non-significant strata, patients with low BubR1 always had the least favourable survival plots. Stratified analyses as performed here, result in smaller numbers in the subgroups. This may have contributed to false, negative results regarding the survival differences of BubR1 expression within some strata. Later studies should therefore test the hypotheses generated in our study regarding different effects of BubR1 within gender, TNM stage, HPV status and p53 status. However, one possible explanation for the difference in survival of patients with tumors with low BubR1 expression within the HPV-negative group, may be that the pathogenetic pathways for HPV-positive and negative carcinomas respectively, are quite different and that BubR1 interacts differently within these pathways.

CONCLUSIONS

TNM and prognostic indices

Previously reported TNM-based stage modifications are all useful predictors of survival in TC. In addition, age, gender, total radiation dose and duration of radiotherapy were important prognostic factors. We propose that both patient-, biological- and treatment factors should be tested carefully when building new prognostic indices in TC.

HPV

This study confirms a high and increasing prevalence of HPV-positive TC (from 38% to 64%) in Norwegian patients. The survival of the HPV-positive group was significantly better only in males. In multivariate analyses HPV-positivity was found to be a significant prognostic factor in addition to age, gender and stage.

BubR1

We have shown that BubR1 expression is a novel and strong prognostic factor in TC, giving additional information to the TNM stage and other known prognostic factors. Low BubR1 expression was correlated with poor prognosis.

FUTURE PERSPECTIVES

The change in etiology of TC due to the increasing prevalence of HPV should be followed up with several actions.

1: In Norway, routine detection of HPV has not been done up to now due to the costs. HPV detection in TC should be included as a routine analysis to follow a possible further increase in prevalence and to facilitate further research.

2: By pooling and reanalyzing the world's 3-4 largest published studies, the HPV and gender interaction in TC could be clarified and settled.

3: A randomized trial should be performed to test the hypothesis that HPV-positive patients with TC can receive less intensive treatment without loss of quality or duration of life.

4: Two questions should be answered: What information should be given and which actions should be taken in males where the female partner has received the diagnosis of a HPV-positive cervical cancer? And the opposite relation; what information should be given and which actions should be taken in women where the male partner has received the diagnosis of a HPV-positive TC?

The spindle protein BubR1 has a high expression and strong impact on prognosis in TC. The expression and importance of spindle proteins in other HNC should therefore be examined. This may provide an essential contribution to understanding the biology of HNC in general. A broader investigation of the impact of spindle protein expression in HNC should also elucidate if TC with high HPV prevalence are outliers in the HNC family. BubR1 expression as a novel and strong prognostic factor in TC should be verified in other patient series.

REFERENCES

1. Akervall J. Genomic screening of head and neck cancer and its implications for therapy planning. Eur Arch Otorhinolaryngol 2006;263: 297-304
2. Akula N, Chen YS, Hennessy K, et al. Utility and accuracy of template-directed dye-terminator incorporation with fluorescence-polarization detection for genotyping single nucleotide polymorphisms. Biotechniques 2002;32: 1072-6, 1078
3. al-Abdulwahed S, Kudryk W, al-Rajhi N, et al. Carcinoma of the tonsil: prognostic factors. J Otolaryngol 1997;26: 296-299
4. Alho OP, Hannula K, Luokkala A, et al. Differential prognostic impact of comorbidity in head and neck cancer. Head Neck 2007;29: 913-918
5. Ando K, Kakeji Y, Kitao H, et al. High expression of BUBR1 is one of the factors for inducing DNA aneuploidy and progression in gastric cancer. Cancer Sci 2009;
6. Andrews E, Shores C, Hayes DN, et al. Concurrent human papillomavirus-associated tonsillar carcinoma in 2 couples. J Infect Dis 2009;200: 882-887
7. Ang KK, Harris J, Wheeler R, et al. Human papillomavirus and survival of patients with oropharyngeal cancer. N Engl J Med 2010;363: 24-35
8. Argiris A, Karamouzis MV, Raben D, et al. Head and neck cancer. Lancet 2008;371: 1695-1709
9. Avissar M, McClean MD, Kelsey KT, et al. MicroRNA expression in head and neck cancer associates with alcohol consumption and survival. Carcinogenesis 2009;30: 2059-2063
10. Baay MF, Quint WG, Koudstaal J, et al. Comprehensive study of several general and type-specific primer pairs for detection of human papillomavirus DNA by PCR in paraffin-embedded cervical carcinomas. J Clin Microbiol 1996;34: 745-747
11. Badaracco G, Venuti A, Bartolazzi A, et al. Overexpression of p53 and bcl-2 proteins and the presence of HPV infection are independent events in head and neck cancer. J Oral Pathol Med 2000;29: 173-179
12. Baker L, Quinlan PR, Patten N, et al. p53 mutation, deprivation and poor prognosis in primary breast cancer. Br J Cancer 2010;102: 719-726
13. Bataini JP, Asselain B, Jaulerry C, et al. A multivariate primary tumour control analysis in 465 patients treated by radical radiotherapy for cancer of the tonsillar region: clinical and treatment parameters as prognostic factors [see comments]. Radiother Oncol 1989;14: 265-277
14. Bentzen SM, Johansen LV, Overgaard J, et al. Clinical radiobiology of squamous cell carcinoma of the oropharynx. Int J Radiat Oncol Biol Phys 1991;20: 1197-1206
15. Berg H. Die prognostische relevanz des TNM-systems für Oropharynxkarzinome. 13 ed 1992:171-177
16. Bernier J, Domenge C, Ozsahin M, et al. Postoperative irradiation with or without concomitant chemotherapy for locally advanced head and neck cancer. N Engl J Med 2004;350: 1945-1952
17. Bertelsen BI, Kugarajh K, Skar R, et al. HPV subtypes in cervical cancer biopsies between 1930 and 2004: detection using general primer pair PCR and sequencing. Virchows Arch 2006;449: 141-147
18. Bhattacharya N, Roy A, Roy B, et al. MYC gene amplification reveals clinical association with head and neck squamous cell carcinoma in Indian patients. J Oral Pathol Med 2009;38: 759-763
19. Bockmuhl U, Petersen I. DNA ploidy and chromosomal alterations in head and neck squamous cell carcinoma. Virchows Arch 2002;441: 541-550
20. Bonner JA, Harari PM, Giralt J, et al. Radiotherapy plus cetuximab for squamous-cell carcinoma of the head and neck. N Engl J Med 2006;354: 567-578
21. Bourhis J, Overgaard J, Audry H, et al. Hyperfractionated or accelerated radiotherapy in head and neck cancer: a meta-analysis. Lancet 2006;368: 843-854
22. Boysen M. Squamous Cell Carcinoma of the Head and Neck in the Elderly. Open Otorhinolyngology Journal 2009;3: 21-27
23. Braakhuis BJ, Brakenhoff RH, Meijer CJ, et al. Human papilloma virus in head and neck cancer: The need for a standardised assay to assess the full clinical importance. Eur J Cancer 2009;

24. Brandsma JL, Abramson AL. Association of papillomavirus with cancers of the head and neck. Arch Otolaryngol Head Neck Surg 1989;115: 621-625
25. Brizel DM. Targeting the future in head and neck cancer. Lancet Oncol 2009;10: 204-205
26. Burum-Auensen E, De Angelis PM, Schjolberg AR, et al. Subcellular localization of the spindle proteins Aurora A, Mad2, and BUBR1 assessed by immunohistochemistry. J Histochem Cytochem 2007;55: 477-486
27. Burum-Auensen E, DeAngelis PM, Schjolberg AR, et al. Spindle proteins Aurora A and BUB1B, but not Mad2, are aberrantly expressed in dysplastic mucosa of patients with longstanding ulcerative colitis. J Clin Pathol 2007;60: 1403-1408
28. Burum-Auensen E, DeAngelis PM, Schjolberg AR, et al. Reduced level of the spindle checkpoint protein BUB1B is associated with aneuploidy in colorectal cancers. Cell Prolif 2008;41: 645-659
29. Califano J, van der RP, Westra W, et al. Genetic progression model for head and neck cancer: implications for field cancerization. Cancer Res 1996;56: 2488-2492
30. Camp RL, Charette LA, Rimm DL. Validation of tissue microarray technology in breast carcinoma. Lab Invest 2000;80: 1943-1949
31. Chan PK, Cheung TH, Tam AO, et al. Biases in human papillomavirus genotype prevalence assessment associated with commonly used consensus primers. Int J Cancer 2006;118: 243-245
32. Charbonneau N, Gelinas M, del VP, et al. Primary radiotherapy for tonsillar carcinoma: a good alternative to a surgical approach. J Otolaryngol 2006;35: 227-234
33. Chen LM, Li G, Reitzel LR, et al. Matched-pair analysis of race or ethnicity in outcomes of head and neck cancer patients receiving similar multidisciplinary care. Cancer Prev Res (Phila Pa) 2009;2: 782-791
34. Chen MH, Chang PM, Chen PM, et al. Prognostic significance of a pretreatment hematologic profile in patients with head and neck cancer. J Cancer Res Clin Oncol 2009;135: 1783-1790
35. Chen R, Aaltonen LM, Vaheri A. Human papillomavirus type 16 in head and neck carcinogenesis. Rev Med Virol 2005;15: 351-363
36. Chien CY, Su CY, Fang FM, et al. Lower prevalence but favorable survival for human papillomavirus-related squamous cell carcinoma of tonsil in Taiwan. Oral Oncol 2008;44: 174-179
37. Chung YL, Lee MY, Horng CF, et al. Use of combined molecular biomarkers for prediction of clinical outcomes in locally advanced tonsillar cancers treated with chemoradiotherapy alone. Head Neck 2009;31: 9-20
38. Cohan DM, Popat S, Kaplan SE, et al. Oropharyngeal cancer: current understanding and management. Curr Opin Otolaryngol Head Neck Surg 2009;17: 88-94
39. Cohen EE, Davis DW, Karrison TG, et al. Erlotinib and bevacizumab in patients with recurrent or metastatic squamous-cell carcinoma of the head and neck: a phase I/II study. Lancet Oncol 2009;10: 247-257
40. Conway DI, Petticrew M, Marlborough H, et al. Socioeconomic inequalities and oral cancer risk: a systematic review and meta-analysis of case-control studies. Int J Cancer 2008;122: 2811-2819
41. Cooper JS, - Farnan NC, - Asbell SO, et al. Recursive partitioning analysis of 2105 patients treated in Radiation Therapy Oncology Group studies of head and neck cancer. - Cancer 1996 May 1;77(9):1905-11 1999; 1905-1911
42. Cripps KJ, Purdie CA, Carder PJ, et al. A study of stabilisation of p53 protein versus point mutation in colorectal carcinoma. Oncogene 1994;9: 2739-2743
43. Curado MP, Hashibe M. Recent changes in the epidemiology of head and neck cancer. Curr Opin Oncol 2009;21: 194-200
44. Custodio AB, Gonzalez-Larriba JL, Bobokova J, et al. Prognostic and predictive markers of benefit from adjuvant chemotherapy in early-stage non-small cell lung cancer. J Thorac Oncol 2009;4: 891-910
45. D'Souza G, Kreimer AR, Viscidi R, et al. Case-control study of human papillomavirus and oropharyngeal cancer. N Engl J Med 2007;356: 1944-1956
46. Dahlstrand H, Nasman A, Romanitan M, et al. Human papillomavirus accounts both for increased incidence and better prognosis in tonsillar cancer. Anticancer Res 2008;28: 1133-1138
47. Dahlstrom KR, Little JA, Zafereo ME, et al. Squamous cell carcinoma of the head and neck in never smoker-never drinkers: a descriptive epidemiologic study. Head Neck 2008;30: 75-84

48. Datema FR, Ferrier MB, van der Schroeff MP, et al. Impact of comorbidity on short-term mortality and overall survival of head and neck cancer patients. Head Neck 2010;32: 728-736
49. Davidson HC, Leibowitz MS, Lopez-Albaitero A, et al. Immunotherapy for head and neck cancer. Oral Oncol 2009;45: 747-751
50. Dayyani F, Etzel CJ, Liu M, et al. Meta-analysis of the impact of human papillomavirus (HPV) on cancer risk and overall survival in head and neck squamous cell carcinomas (HNSCC). Head Neck Oncol 2010;2: 15
51. Dubois JB, Broquerie JL, Delard R, et al. Analysis of the results of irradiation in the treatment of tonsillar region carcinomas. Int J Radiat Oncol Biol Phys 1983;9: 1195-1203
52. El-Mofty SK, Lu DW. Prevalence of human papillomavirus type 16 DNA in squamous cell carcinoma of the palatine tonsil, and not the oral cavity, in young patients: a distinct clinicopathologic and molecular disease entity. Am J Surg Pathol 2003;27: 1463-1470
53. Elashoff JD. Surviving proportional hazards. Hepatology 1983;3: 1031-1035
54. Fakhry C, Gillison ML. Clinical implications of human papillomavirus in head and neck cancers. J Clin Oncol 2006;24: 2606-2611
55. Fakhry C, Westra WH, Li S, et al. Improved survival of patients with human papillomavirus-positive head and neck squamous cell carcinoma in a prospective clinical trial. J Natl Cancer Inst 2008;100: 261-269
56. Fei J, Hong A, Dobbins TA, et al. Prognostic significance of vascular endothelial growth factor in squamous cell carcinomas of the tonsil in relation to human papillomavirus status and epidermal growth factor receptor. Ann Surg Oncol 2009;16: 2908-2917
57. Forastiere A, Koch W, Trotti A, et al. Head and neck cancer. N Engl J Med 2001;345: 1890-1900
58. Forslund O, Nordin P, Hansson BG. Mucosal human papillomavirus types in squamous cell carcinomas of the uterine cervix and subsequently on fingers. Br J Dermatol 2000;142: 1148-1153
59. Fujiwara T, Bandi M, Nitta M, et al. Cytokinesis failure generating tetraploids promotes tumorigenesis in p53-null cells. Nature 2005;437: 1043-1047
60. Gandini S, Botteri E, Iodice S, et al. Tobacco smoking and cancer: a meta-analysis. Int J Cancer 2008;122: 155-164
61. Gao F, Ponte JF, Levy M, et al. hBub1 negatively regulates p53 mediated early cell death upon mitotic checkpoint activation. Cancer Biol Ther 2009;8: 548-556
62. Gerdes J, Schwab U, Lemke H, et al. Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. Int J Cancer 1983;31: 13-20
63. Gillison ML, Koch WM, Capone RB, et al. Evidence for a causal association between human papillomavirus and a subset of head and neck cancers. J Natl Cancer Inst 2000;92: 709-720
64. Gladhaug IP, Westgaard A, Schjolberg AR, et al. Spindle proteins in resected pancreatic head adenocarcinomas: BubR1 is an independent prognostic factor in pancreatobiliary-type tumours. Histopathology 2010;56: 345-355
65. Glombitza F, Guntinas-Lichius O, Petersen I. HPV status in head and neck tumors. Pathol Res Pract 2010;206: 229-234
66. Goon PK, Stanley MA, Ebmeyer J, et al. HPV & head and neck cancer: a descriptive update. Head Neck Oncol 2009;1: 36
67. Goy J, Hall SF, Feldman-Stewart D, et al. Diagnostic delay and disease stage in head and neck cancer: a systematic review. Laryngoscope 2009;119: 889-898
68. Ha GH, Baek KH, Kim HS, et al. p53 activation in response to mitotic spindle damage requires signaling via BubR1-mediated phosphorylation. Cancer Res 2007;67: 7155-7164
69. Haddad R, Crum C, Chen Z, et al. HPV16 transmission between a couple with HPV-related head and neck cancer. Oral Oncol 2008;44: 812-815
70. Hafkamp HC, Manni JJ, Haesevoets A, et al. Marked differences in survival rate between smokers and nonsmokers with HPV 16-associated tonsillar carcinomas. Int J Cancer 2008;122: 2656-2664
71. Hafkamp HC, Mooren JJ, Claessen SM, et al. P21 Cip1/WAF1 expression is strongly associated with HPV-positive tonsillar carcinoma and a favorable prognosis. Mod Pathol 2009;22: 686-698
72. Hall SF, Groome PA, Irish J, et al. Towards further understanding of prognostic factors for head and neck cancer patients: the example of hypopharyngeal cancer. Laryngoscope 2009;119: 696-702

73. Hall SF, Groome PA, Rothwell D, et al. Using tnm staging to predict survival in patients with squamous cell carcinoma of head & neck. Head Neck J Sci Spec Head Nec 1999;21: 30-38:
74. Hansson BG, Rosenquist K, Antonsson A, et al. Strong association between infection with human papillomavirus and oral and oropharyngeal squamous cell carcinoma: a population-based case-control study in southern Sweden. Acta Otolaryngol 2005;125: 1337-1344
75. Harmer M. TNM classification of malignant tumours. UICC; 1978
76. Hart AA, Mak-Kregar S, Hilgers FJ, et al. The importance of correct stage grouping in oncology. Results of a nationwide study of oropharyngeal carcinoma in The Netherlands. Cancer 1995;75: 2656-2662
77. Hashibe M, Brennan P, Benhamou S, et al. Alcohol drinking in never users of tobacco, cigarette smoking in never drinkers, and the risk of head and neck cancer: pooled analysis in the International Head and Neck Cancer Epidemiology Consortium. J Natl Cancer Inst 2007;99: 777-789
78. Hass HG, Schmidt A, Nehls O, et al. DNA ploidy, proliferative capacity and intratumoral heterogeneity in primary and recurrent head and neck squamous cell carcinomas (HNSCC)--potential implications for clinical management and treatment decisions. Oral Oncol 2008;44: 78-85
79. Hemmer J, Kraft K. Stability of aneuploid clones during oral squamous cell carcinoma metastasis. Anticancer Res 2001;21: 1459-1464
80. Hemminki K, Dong C, Frisch M. Tonsillar and other upper aerodigestive tract cancers among cervical cancer patients and their husbands. Eur J Cancer Prev 2000;9: 433-437
81. Heng B, Glenn WK, Ye Y, et al. Human papilloma virus is associated with breast cancer. Br J Cancer 2009;101: 1345-1350
82. Hermanek P, Hutter RV, Sobin LH. Prognostic grouping: the next step in tumor classification. J Cancer Res Clin Oncol 1990;116: 513-516
83. Hermanek P, Sobin LH, Fleming ID. What do we need beyond TNM? [editorial]. Cancer 1996;77: 815-817
84. Herrero R, Castellsague X, Pawlita M, et al. Human papillomavirus and oral cancer: the International Agency for Research on Cancer multicenter study. J Natl Cancer Inst 2003;95: 1772-1783
85. Hilsenbeck SG, Clark GM, McGuire WL. Why do so many prognostic factors fail to pan out? Breast Cancer Res Treat 1992;22: 197-206
86. Hiraki A, Matsuo K, Suzuki T, et al. Teeth loss and risk of cancer at 14 common sites in Japanese. Cancer Epidemiol Biomarkers Prev 2008;17: 1222-1227
87. Hobbs CG, Sterne JA, Bailey M, et al. Human papillomavirus and head and neck cancer: a systematic review and meta-analysis. Clin Otolaryngol 2006;31: 259-266
88. Hoos A, Urist MJ, Stojadinovic A, et al. Validation of tissue microarrays for immunohistochemical profiling of cancer specimens using the example of human fibroblastic tumors. Am J Pathol 2001;158: 1245-1251
89. Hsieh PC, Chen YK, Tsai KB, et al. Expression of BUBR1 in human oral potentially malignant disorders and squamous cell carcinoma. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2010;109: 257-267
90. Izumi H, Matsumoto Y, Ikeuchi T, et al. BubR1 localizes to centrosomes and suppresses centrosome amplification via regulating Plk1 activity in interphase cells. Oncogene 2009;28: 2806-2820
91. Jaber JJ, Moreira J, Canar WJ, et al. A 25-year analysis of veterans treated for tonsillar squamous cell carcinoma. Arch Otolaryngol Head Neck Surg 2009;135: 1147-1153
92. Jallepalli PV, Lengauer C. Chromosome segregation and cancer: cutting through the mystery. Nat Rev Cancer 2001;1: 109-117
93. Jeremic B, Milicic B. Pretreatment prognostic factors of survival in patients with locally advanced nonmetastatic squamous cell carcinoma of the head and neck treated with radiation therapy with or without concurrent chemotherapy. Am J Clin Oncol 2009;32: 163-168
94. Jones AS, Beasley N, Houghton D, et al. The effects of age on survival and other parameters in squamous cell carcinoma of the oral cavity, pharynx and larynx. Clin Otolaryngol Allied Sci 1998;23: 51-56
95. Jones GW, Browman G, Goodyear M, et al. Comparison of the addition of T and N integer scores with TNM stage groups in head and neck cancer. Head Neck 1993;15: 497-503

96. Juhasz A, Balazs M, Sziklay I, et al. Chromosomal imbalances in laryngeal and hypopharyngeal cancers detected by comparative genomic hybridization. Cytometry A 2005;67: 151-160
97. Kaplan EL, Meier P. Nonparametric estimation from incomplete observations. J Amer Statist Assoc 1958;53: 457-481
98. Kerr A. Scott-Brown's Otolaryngology. 5th ed. Butterworth; 1987
99. Kleter B, van Doorn LJ, Schrauwen L, et al. Development and clinical evaluation of a highly sensitive PCR-reverse hybridization line probe assay for detection and identification of anogenital human papillomavirus. J Clin Microbiol 1999;37: 2508-2517
100. Kong CS, Narasimhan B, Cao H, et al. The relationship between human papillomavirus status and other molecular prognostic markers in head and neck squamous cell carcinomas. Int J Radiat Oncol Biol Phys 2009;74: 553-561
101. Kononen J, Bubendorf L, Kallioniemi A, et al. Tissue microarrays for high-throughput molecular profiling of tumor specimens. Nat Med 1998;4: 844-847
102. Kops GJ, Weaver BA, Cleveland DW. On the road to cancer: aneuploidy and the mitotic checkpoint. Nat Rev Cancer 2005;5: 773-785
103. Kumar RV, Kadkol SS, Daniel R, et al. Human papillomavirus, p53 and cyclin D1 expression in oropharyngeal carcinoma. Int J Oral Maxillofac Surg 2003;32: 539-543
104. Kunicka Z, Mucha I, Fajkus J. Telomerase activity in head and neck cancer. Anticancer Res 2008;28: 3125-3129
105. Kwok J, Langevin SM, Argiris A, et al. The impact of health insurance status on the survival of patients with head and neck cancer. Cancer 2010;116: 476-485
106. Lacy PD, Piccirillo JF, Merritt MG, et al. Head and neck squamous cell carcinoma: better to be young. Otolaryngol Head Neck Surg 2000;122: 253-258
107. Lango MN. Multimodal treatment for head and neck cancer. Surg Clin North Am 2009;89: 43-52, viii
108. Lee YK, Choi E, Kim MA, et al. BubR1 as a prognostic marker for recurrence-free survival rates in epithelial ovarian cancers. Br J Cancer 2009;101: 504-510
109. Leemans CR, Braakhuis BJ, Brakenhoff RH. The molecular biology of head and neck cancer. Nat Rev Cancer 2011;11: 9-22
110. Lengauer C, Kinzler KW, Vogelstein B. Genetic instability in colorectal cancers. Nature 1997;386: 623-627
111. Lewin F, Damber L, Jonsson H, et al. Neoadjuvant chemotherapy with cisplatin and 5-fluorouracil in advanced squamous cell carcinoma of the head and neck: a randomized phase III study. Radiotherapy & Oncology 1997;43: 23-28
112. Li R, Sonik A, Stindl R, et al. Aneuploidy vs. gene mutation hypothesis of cancer: recent study claims mutation but is found to support aneuploidy. Proc Natl Acad Sci U S A 2000;97: 3236-3241
113. Li W, Tran N, Lee SC, et al. New evidence for geographic variation in the role of human papillomavirus in tonsillar carcinogenesis. Pathology 2007;39: 217-222
114. Lindelov B, Monberg J, Sand H. Squamous cell carcinoma of the oropharynx. Results of primary radiotherapy. Acta Oncologica 1992;31: 341-345
115. Lira RC, Miranda FA, Guimaraes MC, et al. BUBR1 expression in benign oral lesions and squamous cell carcinomas: Correlation with human papillomavirus. Oncol Rep 2010;23: 1027-1036
116. Lonning PE. Breast cancer prognostication and prediction: are we making progress? Ann Oncol 2007;18 Suppl 8: viii3-viii7
117. Lydiatt WM, Shah JP, Hoffman HT, et al. AJCC stage groupings for head and neck cancer: should we look at alternatives? A report of the Head and Neck Sites Task Force. Head & Neck 2001;23: 607-612
118. Machtay M, Rosenthal DI, Hershock D, et al. Organ preservation therapy using induction plus concurrent chemoradiation for advanced resectable oropharyngeal carcinoma: a University of Pennsylvania Phase II Trial. J Clin Oncol 2002;20: 3964-3971
119. Mantravadi RV, Liebner EJ, Ginde JV. An analysis of factors in the successful management of cancer of tonsillar region. Cancer 1978;41: 1054-1058
120. Marron M, Boffetta P, Zhang ZF, et al. Cessation of alcohol drinking, tobacco smoking and the reversal of head and neck cancer risk. Int J Epidemiol 2010;39: 182-196
121. Marx J. Debate surges over the origins of genomic defects in cancer. Science 2002;297: 544-546

122. McCaul JA, Gordon KE, Clark LJ, et al. Telomerase inhibition and the future management of head-and-neck cancer. Lancet Oncol 2002;3: 280-288
123. Mell LK, Dignam JJ, Salama JK, et al. Predictors of competing mortality in advanced head and neck cancer. J Clin Oncol 2010;28: 15-20
124. Mellin H, Friesland S, Auer G, et al. Human papillomavirus and DNA ploidy in tonsillar cancer--correlation to prognosis. Anticancer Res 2003;23: 2821-2828
125. Mendenhall WM, Logan HL. Human Papillomavirus and Head and Neck Cancer. Am J Clin Oncol 2009;
126. Mendenhall WM, Parsons JT, Cassisi NJ, et al. Squamous cell carcinoma of the tonsillar area treated with radical irradiation. Radiother Oncol 1987;10: 23-30
127. Mendenhall WM, Parsons JT, Mendenhall NP, et al. Brachytherapy in head and neck cancer: selection criteria and results at the University of Florida. Oncology Huntingt 1991;5: 44-54
128. Minhas KM, Singh B, Jiang WW, et al. Spindle assembly checkpoint defects and chromosomal instability in head and neck squamous cell carcinoma. Int J Cancer 2003;107: 46-52
129. Mork J, Lie AK, Glattre E, et al. Human papillomavirus infection as a risk factor for squamous-cell carcinoma of the head and neck. N Engl J Med 2001;344: 1125-1131
130. Nasman A, Attner P, Hammarstedt L, et al. Incidence of human papillomavirus (HPV) positive tonsillar carcinoma in Stockholm, Sweden: an epidemic of viral-induced carcinoma? Int J Cancer 2009;125: 362-366
131. National Comprehensive Cancer Network. Clinical Practice Guidelines in Oncology. Head and neck cancer. www.nccs.org. 2010.
132. Nguyen NP, Chi A, Nguyen LM, et al. Human papillomavirus-associated oropharyngeal cancer: a new clinical entity. QJM 2010;103: 229-236
133. Nordsmark M, Bentzen SM, Rudat V, et al. Prognostic value of tumor oxygenation in 397 head and neck tumors after primary radiation therapy. An international multi-center study. Radiother Oncol 2005;77: 18-24
134. Overgaard J, Hansen HS, Overgaard M, et al. A randomized double-blind phase III study of nimorazole as a hypoxic radiosensitizer of primary radiotherapy in supraglottic larynx and pharynx carcinoma. Results of the Danish Head and Neck Cancer Study (DAHANCA) Protocol 5-85. Radiother Oncol 1998;46: 135-146
135. Overgaard J, Hansen HS, Specht L, et al. Five compared with six fractions per week of conventional radiotherapy of squamous-cell carcinoma of head and neck: DAHANCA 6 and 7 randomised controlled trial. Lancet 2003;362: 933-940
136. Pancione M, Forte N, Fucci A, et al. Prognostic role of beta-catenin and p53 expression in the metastatic progression of sporadic colorectal cancer. Hum Pathol 2010;41: 867-876
137. Parsons JT, Mendenhall WM, Stringer SP, et al. Squamous cell carcinoma of the oropharynx: surgery, radiation therapy, or both. Cancer 2002;94: 2967-2980
138. Perez-Ordonez B, Beauchemin M, Jordan RC. Molecular biology of squamous cell carcinoma of the head and neck. J Clin Pathol 2006;59: 445-453
139. Peters ES, Luckett BG, Applebaum KM, et al. Dairy products, leanness, and head and neck squamous cell carcinoma. Head Neck 2008;30: 1193-1205
140. Peto R, Pike MC, Armitage P, et al. Design and analysis of randomized clinical trials requiring prolonged observation for each patient. II. Analysis and examples. Br J Cancer 1977;35: 1-39
141. Pignon JP, Bourhis J, Domenge C, et al. Chemotherapy added to locoregional treatment for head and neck squamous-cell carcinoma: three meta-analyses of updated individual data. MACH-NC Collaborative Group. Meta-Analysis of Chemotherapy on Head and Neck Cancer. Lancet 2000;355: 949-955
142. Pignon JP, le MA, Maillard E, et al. Meta-analysis of chemotherapy in head and neck cancer (MACH-NC): an update on 93 randomised trials and 17,346 patients. Radiother Oncol 2009;92: 4-14
143. Pinto M, Vieira J, Ribeiro FR, et al. Overexpression of the mitotic checkpoint genes BUB1 and BUBR1 is associated with genomic complexity in clear cell kidney carcinomas. Cell Oncol 2008;30: 389-395
144. Qu W, Jiang G, Cruz Y, et al. PCR detection of human papillomavirus: comparison between MY09/MY11 and GP5+/GP6+ primer systems. J Clin Microbiol 1997;35: 1304-1310

145. Ragin CC, Taioli E. Survival of squamous cell carcinoma of the head and neck in relation to human papillomavirus infection: review and meta-analysis. Int J Cancer 2007;121: 1813-1820
146. Rahmani Z, Gagou ME, Lefebvre C, et al. Separating the spindle, checkpoint, and timer functions of BubR1. J Cell Biol 2009;187: 597-605
147. Ritchie JM, Smith EM, Summersgill KF, et al. Human papillomavirus infection as a prognostic factor in carcinomas of the oral cavity and oropharynx. Int J Cancer 2003;104: 336-344
148. Rizzardi C, Torelli L, Barresi E, et al. BUBR1 expression in oral squamous cell carcinoma and its relationship to tumor stage and survival. Head Neck 2010; Nov 10 [epub ahead of print]
149. Robinson M, Sloan P, Shaw R. Refining the diagnosis of oropharyngeal squamous cell carcinoma using human papillomavirus testing. Oral Oncol 2010;46: 492-496
150. Romanitan M, Nasman A, Ramqvist T, et al. Human papillomavirus frequency in oral and oropharyngeal cancer in Greece. Anticancer Res 2008;28: 2077-2080
151. Rosenquist K. Risk factors in oral and oropharyngeal squamous cell carcinoma: a population-based case-control study in southern Sweden. Swed Dent J Suppl 2005; 1-66
152. Rosenquist K, Wennerberg J, Annertz K, et al. Recurrence in patients with oral and oropharyngeal squamous cell carcinoma: human papillomavirus and other risk factors. Acta Otolaryngol 2007;127: 980-987
153. Rowan K. Should cetuximab replace cisplatin in head and neck cancer? J Natl Cancer Inst 2010;102: 74-6, 78
154. Sarafoleanu D, Postelnicu V, Iosif C, et al. The role of p53, PCNA and Ki-67 as outcome predictors in the treatment of laryngeal cancer. J Med Life 2009;2: 219-226
155. Sather HN. The use of prognostic factors in clinical trials. Cancer 1986;58: 461-467
156. Scintu M, Vitale R, Prencipe M, et al. Genomic instability and increased expression of BUB1B and MAD2L1 genes in ductal breast carcinoma. Cancer Lett 2007;254: 298-307
157. Shah NG, Trivedi TI, Tankshali RA, et al. Prognostic significance of molecular markers in oral squamous cell carcinoma: a multivariate analysis. Head Neck 2009;31: 1544-1556
158. Shukla S, Bharti AC, Mahata S, et al. Infection of human papillomaviruses in cancers of different human organ sites. Indian J Med Res 2009;130: 222-233
159. Shukovsky LJ, Fletcher GH. Time-dose and tumor volume relationships in the irradiation of squamous cell carcinoma of the tonsillar fossa. Radiology 1973;107: 621-626
160. Shuman AG, Wolf GT. Human papillomavirus status in head and neck cancer: the ethics of disclosure. Cancer 2010;116: 4221-4226
161. Sieber OM, Heinimann K, Tomlinson IP. Genomic instability--the engine of tumorigenesis? Nat Rev Cancer 2003;3: 701-708
162. Skov BG, Holm B, Erreboe A, et al. ERCC1 and Ki67 in Small Cell Lung Carcinoma and Other Neuroendocrine Tumors of the Lung: Distribution and Impact on Survival. J Thorac Oncol 2010;5: 453-459
163. Smeets SJ, Hesselink AT, Speel EJ, et al. A novel algorithm for reliable detection of human papillomavirus in paraffin embedded head and neck cancer specimen. Int J Cancer 2007;121: 2465-2472
164. Smith BD, Smith GL, Carter D, et al. Prognostic significance of vascular endothelial growth factor protein levels in oral and oropharyngeal squamous cell carcinoma. J Clin Oncol 2000;18: 2046-2052
165. Smith EM, Ritchie JM, Summersgill KF, et al. Age, sexual behavior and human papillomavirus infection in oral cavity and oropharyngeal cancers. Int J Cancer 2004;108: 766-772
166. Smith EM, Rubenstein LM, Hoffman H, et al. Human papillomavirus, p16 and p53 expression associated with survival of head and neck cancer. Infect Agent Cancer 2010;5: 4
167. Snyderman CH, - Wagner RL. - Superiority of the T and N integer score (TANIS) staging system for squamous cell carcinoma of the oral cavity. - Otolaryngology - Head & Neck Surgery 1995 Jun;112(6):691-4 1999; 691-694
168. Spiessl Bea. TNM Atlas. UICC; 1989
169. Syrjanen K, Syrjanen S, Lamberg M, et al. Morphological and immunohistochemical evidence suggesting human papillomavirus (HPV) involvement in oral squamous cell carcinogenesis. Int J Oral Surg 1983;12: 418-424

170. Syrjanen S. HPV infections and tonsillar carcinoma. J Clin Pathol 2004;57: 449-455
171. Syrjanen S. The role of human papillomavirus infection in head and neck cancers. Ann Oncol 2010;21 Suppl 7: vii243-vii245
172. Thames HD, Bentzen SM. Time factor for tonsillar carcinoma [editorial; comment]. Int J Radiat Oncol Biol Phys 1995;33: 755-758
173. Tibshirani R. A plain man's guide to the proportional hazards model. Clin Invest Med 1982;5: 63-68
174. Veltman JA, Bot FJ, Huynen FC, et al. Chromosome instability as an indicator of malignant progression in laryngeal mucosa. J Clin Oncol 2000;18: 1644-1651
175. Vermorken JB, Mesia R, Rivera F, et al. Platinum-based chemotherapy plus cetuximab in head and neck cancer. N Engl J Med 2008;359: 1116-1127
176. Withers HR, Taylor JM, Maciejewski B. The hazard of accelerated tumor clonogen repopulation during radiotherapy. Acta Oncol 1988;27: 131-146
177. Yamamoto Y, Matsuyama H, Chochi Y, et al. Overexpression of BUBR1 is associated with chromosomal instability in bladder cancer. Cancer Genet Cytogenet 2007;174: 42-47
178. Yang ES, Murphy BM, Chung CH, et al. Evolution of clinical trials in head and neck cancer. Crit Rev Oncol Hematol 2009;71: 29-42
179. Ye YK, Bi XC, He HC, et al. CK20 and Ki-67 as significant prognostic factors in human bladder carcinoma. Clin Exp Med 2010;10: 153-158
180. Yerushalmi R, Woods R, Ravdin PM, et al. Ki67 in breast cancer: prognostic and predictive potential. Lancet Oncol 2010;11: 174-183
181. Young LS, Murray PG. Epstein-Barr virus and oncogenesis: from latent genes to tumours. Oncogene 2003;22: 5108-5121
182. zur Hausen H. Papillomaviruses in the causation of human cancers - a brief historical account. Virology 2009;384: 260-265